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Streptomycin Action and Anaerobiosis

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SUMMARY

The question was asked whether the insensitivity to dihydrostreptomycin and streptomycin exhibited by facultatively anaerobic organisms growing strictly anaerobically could be due entirely to the lack of an anaerobic mechanism for the uptake of these antibiotics. A technique is described here which allows exposure of *Escherichia coli* B to dihydrostreptomycin, under conditions which are known to promote its intracellular accumulation, for a time, followed by further incubation in a growth medium under aerobic as well as under strictly anaerobic conditions, after removal of the extracellular antibiotic. With this technique it was possible to make a quantitative comparison of the inhibitory effects of dihydrostreptomycin on aerobic and anaerobic growth of *E. coli* B under conditions where no further uptake of the antibiotic could occur. The results show that for any given treatment of *E. coli* B with dihydrostreptomycin, subsequent aerobic and anaerobic growth rates are inhibited to exactly the same extent. It is concluded that the need for aerobic metabolism in the expression of antibiotic effect by dihydrostreptomycin is concerned only with its uptake into the organisms.

INTRODUCTION

In a review Kogut & Lightbown (1964*a*) pointed out that the current hypothesis about the mode of action of streptomycin, namely that it inhibits protein synthesis (Erdös & Ullmann, 1959; Hahn *et al.* 1962) by combining with the ribosomes of sensitive organisms (Spotts & Stanier, 1961; Spotts, 1962; Flaks, Cox & White, 1962; Speyer, Lengyel & Basilio, 1962; Mager, Benedict & Artman, 1962) leaves unexplained the frequently reported observation that strictly anaerobic bacteria and facultatively anaerobic bacteria when growing anaerobically are insensitive to the drug (Lightbown, 1957). However, Hancock (1962*a*) in a study of the intracellular accumulation of streptomycin, showed that no such accumulation took place when facultatively anaerobic organisms were grown under conditions which inhibited aerobic respiration. It seemed possible, therefore, that this dependence on aerobic metabolism of the uptake of streptomycin (and dihydrostreptomycin) into the organism might account for the necessity of aerobic conditions for antibiotic activity of the drug to be expressed. Since it is now known that several factors influence the uptake and intracellular accumulation of streptomycin (Hancock, 1962*a*, *b*; Hurwitz & Rosano, 1962) and since such intracellular accumulation is presumably a necessary but by no means a sufficient condition for the antibiotic action, it seemed desirable to use experimental systems in which the entry of streptomycin into the cell could be dissociated from its further effects, to see

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whether the effect of anaerobiosis is solely on uptake. The present paper describes a technique for achieving this, and compares the growth-inhibitory effect of dihydrostreptomycin on a sensitive facultative bacterial strain during aerobic and anaerobic growth, subsequent to antibiotic treatments which are known to promote its intracellular accumulation by the organisms. Parts of this work have been communicated previously (Kogut & Lightbown, 1963; Kogut & Lightbown, 1964*b*).

METHODS

Organism and growth conditions. The organism used was *Escherichia coli* B (obtained from Dr M. Pollock, National Institute for Medical Research) which was kept at 4° on Hedley Wright agar slopes (Wright, 1933) after growth for 18 hr at 37°, and subcultured weekly. For experiments the growth medium was that of McQuillen & Roberts (1954) modified by the addition before autoclaving of 0·2 % (w/v) trisodium citrate. A 10 % (w/v) glucose solution (sterilized by filtration) was added at the time of inoculation to give a final concentration of 0·2 % (w/v) glucose. The temperature of incubation was 32°. Growth was measured as extinction (E) at 500 mμ (E_{500}) in a Hilger 'Uvispeck' spectrophotometer with optical cells of 5 mm. light path, and recorded as logarithms to the base 2 (Finney, Hazlewood & Smith, 1955). Standard inocula were prepared as follows. The growth from one agar slope was suspended in 1 ml. of sterile growth medium, and 0·2 ml. of this suspension added to 50 ml. sterile medium in a 250 ml. Erlenmeyer flask. This inoculum was allowed to grow at 32° for 6–10 hr, or another logarithmically growing culture was used. The extinction (E_{500}) was measured, and a portion of the culture diluted with medium to a calculated E_{500} of 0·001. Of this suspension, 1·0 ml./50 ml. fresh growth medium gave cultures in the logarithmic phase after incubation for 16–18 hr at 32°. Aerobiosis was achieved by shaking flasks in a constant temperature waterbath at 120 strokes/min. (throw 4·5 cm.); under these conditions it was determined experimentally that up to 60 ml. culture in a 250 ml. Erlenmeyer flask, and up to 300 ml. in a 1 l. Erlenmeyer flask gave adequate aeration.

Strictly anaerobic growth under conditions where samples could be withdrawn for E_{500} measurements without admitting air to the culture, was achieved as follows (Fig. 1). The needle of a 30 ml. all-glass hypodermic syringe was replaced with a length of capillary tubing (glass and silicone rubber) and the end of this tubing was closed with a spring clip. The assembled apparatus, wrapped in paper, was sterilized by autoclaving (20 min., 121°). When needed, the plunger was withdrawn, the culture introduced into the barrel, the plunger replaced and all gas expelled until the capillary tubing was filled with culture. The filled syringes were held in the water-bath by means of spring clips mounted on a board so that the whole of the culture in the syringe and most of that in the capillary tubing was immersed. Samples, when necessary, were withdrawn by depressing the plunger and opening the capillary tubing. The first 0·5 ml. of each sample was always discarded. Anaerobic additions to such cultures could be made by injection through the silicone tubing into the barrel of the syringe from another syringe; mixing was achieved by drawing the culture into the injecting syringe a number of times.

Total counts were made by using a Thoma counting chamber of depth 0·02 mm.; a minimum of 400 organisms/sample were counted.

Dry weights were determined by drying appropriate samples, after washing 3 times with water by centrifugation, in aluminium foil 'boats' on a hot plate at 60° and leaving in a desiccator over P_2O_5 until the weights were constant.

Filtration treatment. Rapid transfer of growing organisms from one medium to another was achieved by filtration on membrane filters (Millipore; diameter 47 mm.; A.P.D., 0·22 μ) followed by washing with and resuspension in fresh medium pre-warmed to the growth temperature.

Assays of dihydrostreptomycin concentrations were by the diffusion method (Humphrey & Lightbown, 1952). Dihydrostreptomycin sulphate, potency 800 i.u./mg. was obtained from Distillers (Biochemicals) Co. Ltd. Speke, Liverpool 1.

Chemicals used were all A.R. grade.

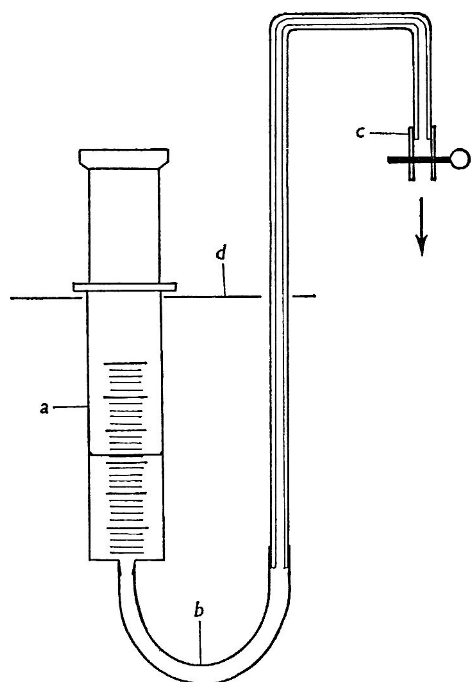

Fig. 1

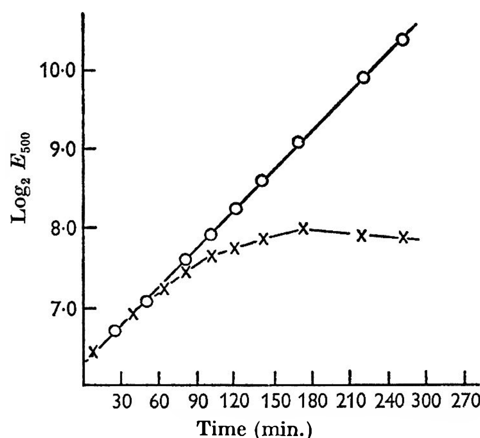

Fig. 2

Fig. 1. Syringe for anaerobic growth of *E. coli* B. (*a*) 30 ml. all glass-syringe half filled with culture. (*b*) Silicone rubber tubing connecting syringe with capillary glass tubing. (*c*) Silicone rubber tubing carrying spring-clip for withdrawing samples. (*d*) Level of water bath relative to barrel of syringe and capillary tubing.

Fig. 2. Growth curve of *E. coli* B, without, and after addition of, 20 μg./ml. dihydro-streptomycin. Growth medium and temperature as described in 'Methods'. E_{500} in cuvettes of 5 mm. light path, plotted as $\log_2$ on the ordinate. ○ – ○ = Control. × – × = +20 μg. dihydrostreptomycin sulphate/ml. added at 0 min.

RESULTS

Under the experimental conditions described the specific growth rates, μ (Herbert, Elsworth & Telling, 1956) of *Escherichia coli* B were approximately 1·0 (μ = doublings/hr). Addition of 20 μg. dihydrostreptomycin sulphate/ml. culture

during the early exponential stage of aerobic growth (E_{500} 0·1–0·2) produced a time course of growth inhibition as shown in Fig. 2. Table 1 shows the relationships between E_{500} measurements and total counts and dry-weight determinations for samples taken at different times, with and without dihydrostreptomycin. The results show that there was essentially no change in the ratios E_{500}:dry-weight and total count up to the time when growth inhibition was complete, thus justifying the use of E_{500} determinations to measure inhibition of growth.

Table 1. *Ratio of total counts and dry weights to E_{500} of E. coli* B *cultures growing in the presence and absence of dihydrostreptomycin*

	Controls				+20 μg. dihydrostreptomycin sulphate/ml. at 0 min.				
Expt. no.	Time (min.)	E_{500}	Total count/ E_{500}	mg. dry wt./ E_{500}	Time (min.)	E_{500}	% Inhibition of μ	Total count/ E_{500}	mg. dry wt./ E_{500}
1	15	0·115	1·74 × 10⁹	—	15	0·116	0	1·78 × 10⁹	—
	30	0·136	1·66 × 10⁹	—	45	0·161	0	1·93 × 10⁹	—
	60	0·192	1·70 × 10⁹	—	60	0·186	17	1·72 × 10⁹	—
	85	0·251	2·08 × 10⁹	—	95	0·240	45	2·07 × 10⁹	0·530
	110	0·349	—	0·565	110	0·258	60	1·68 × 10⁹	—
	115	0·349	1·68 × 10⁹	—	130	0·281	64	1·80 × 10⁹	—
	185	0·740	—	0·565	145	0·274	92	2·02 × 10⁹	—
					162	0·287	96	1·94 × 10⁹	—
2	46	0·227	1·84 × 10⁹	—	30	0·185	0	1·55 × 10⁹	—
	85	0·349	1·41 × 10⁹	—	60	0·246	13	1·44 × 10⁹	—
	120	0·540	1·64 × 10⁹	—	85	0·309	29	1·86 × 10⁹	0·563
	193	1·164	1·67 × 10⁹	0·590	100	0·340	42·5	2·17 × 10⁹	—
					120	0·375	56	1·67 × 10⁹	0·555
3	20	0·101	1·83 × 10⁹	—	80	0·241	48	—	0·526
	185	0·990	—	0·560	130	0·288	88	1·58 × 10⁹	0·545
					145	0·290	100	1·41 × 10⁹	—
					165	0·291	100	1·36 × 10⁹	—
					185	0·294	100	1·37 × 10⁹	—
					255	0·260	100	1·45 × 10⁹	—

In preliminary experiments a culture of *Escherichia coli* B growing aerobically in the early logarithmic phase, was divided into two flasks, A and B. From flask A, two samples each of 30 ml. were withdrawn and placed in syringes for anaerobic growth, at the same time as dihydrostreptomycin sulphate 20 μg./ml. was added to the flask B. Of the two anaerobic controls, one received no antibiotic, and to the other dihydrostreptomycin sulphate 20 μg./ml. was added, by injecting it through the silicone rubber tube, at a time when it was estimated from Q_{O_2} and extinction measurements that anaerobic growth should have been established. From flask B, samples were withdrawn at various times after the addition of dihydrostreptomycin and placed into syringes to grow anaerobically. Figure 3*a* shows that when dihydrostreptomycin was added to a culture growing anaerobically, growth was unaffected. However (Fig. 3*b*) when a culture was made anaerobic at various times after the addition of dihydrostreptomycin, growth continued apparently exponentially, though at rates which were slower as compared with the anaerobic controls. The extent of inhibition appeared to be proportional to the duration of previous aerobic growth in the presence of dihydrostreptomycin.

There were, however, difficulties in the quantitative interpretation of these results. First, at the low organism concentrations used, it always took 10–20 min. for the organisms to exhaust the dissolved oxygen in the medium and thus to establish strictly anaerobic conditions; during this period further uptake of dihydrostreptomycin might have occurred. Secondly, and more important, there was always a lag period during which no change in extinction occurred, before anaerobic growth became established (see Fig. 3*a*, *b*). It might be argued therefore that the

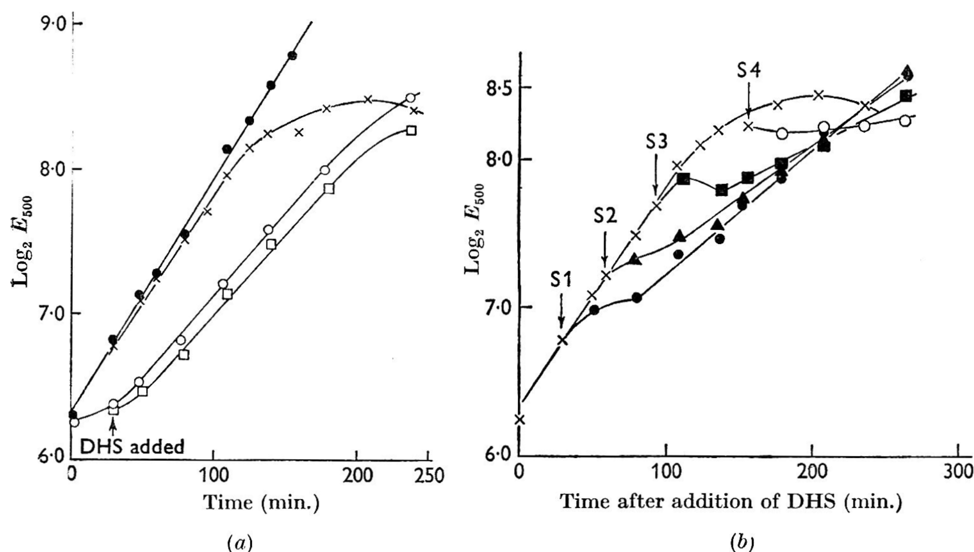

Fig. 3. Aerobic and anaerobic growth of *Escherichia coli* B after addition of 20 μg. dihydrostreptomycin sulphate/ml. (DHS). Growth medium and temperature as in 'Methods'. (*a*) ●—● = Control growing aerobically. ×—× = Aerobic growth after addition of dihydrostreptomycin at 0 min. ○—○ = Sample removed from control at 0 min. and transferred to anaerobic growth. □—□ = Sample removed from control at 0 min., transferred to anaerobic growth and dihydrostreptomycin added at arrow. (*b*) ×—× = Parent culture growing aerobically in presence of dihydrostreptomycin added at 0 min. ●—● = S1, ▲—▲ = S2, ■—■ = S3, ○—○ = S4, samples removed at various times after addition of dihydrostreptomycin, indicated by arrows, from aerobic parent culture, and transferred to anaerobic conditions.

lower rates of anaerobic growth, after treatment of the organisms with dihydrostreptomycin under aerobic conditions, might have been due to an inhibitory effect of the antibiotic, inside the organisms, on an adaptive process required to allow anaerobic growth (Fowler, 1951) and not to an effect on anaerobic growth itself. This possibility was eliminated by growing the organisms anaerobically for two generations or more before making them aerobic and adding the dihydrostreptomycin. After such a period of previous anaerobic growth, followed by a limited period of aerobic growth in the presence of dihydrostreptomycin, the next change-over to anaerobiosis occurred without a lag in the growth curve.

A further modification of the experimental technique made possible a direct quantitative comparison between the degree of inhibition of aerobic and anaerobic growth. Samples withdrawn from a parent culture growing aerobically in the

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presence of dihydrostreptomycin were filtered on membranes, these washed twice with the original volume of pre-warmed antibiotic-free growth medium to remove the free as well as most of the adsorbed dihydrostreptomycin (Hancock, 1962*a*;

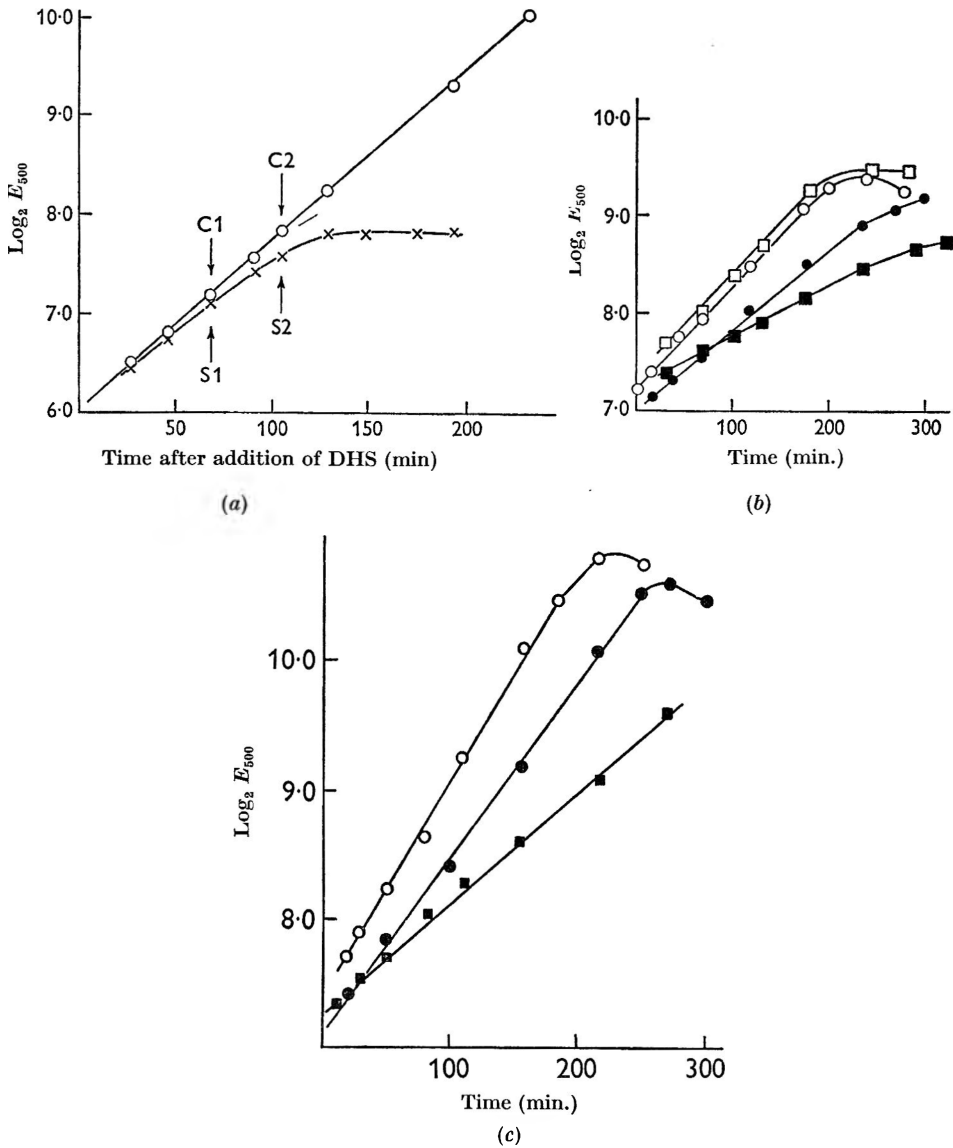

Fig. 4. Effect of 20 μg. dihydrostreptomycin sulphate/ml. during aerobic exponential growth on subsequent aerobic and anaerobic growth in the absence of extracellular antibiotic. (*a*) Parent cultures: ○—○ = control, ×—× = + dihydrostreptomycin. (*b*) Anaerobic growth of samples removed from parent cultures at times indicated by arrows (in (*a*)) and freed of extracellular antibiotic by filtration and washing. Open symbols = controls, filled symbols = treated culture; ●—●. ○—○ = S1; ■—■. □—□ = S2. (*c*) Aerobic growth of samples removed from parent cultures at times indicated by arrows (in (*a*)) and freed of extracellular antibiotic by filtration and washing. Open symbols = control, filled symbols = treated culture; ●—●. ○—○ = S1; ■—■ = S2.

Hurwitz & Rosano, 1962) and then the organisms resuspended in the original volume of warm antibiotic-free medium. Each resuspended sample was divided into two equal portions of which one was grown aerobically and the other anaerobically. Sensitive antibiotic assays of the washings and resuspending medium, collected separately, showed that less than 0·10 μg. free dihydrostreptomycin/ml. was carried over into the final resuspended culture. The time taken from withdrawal

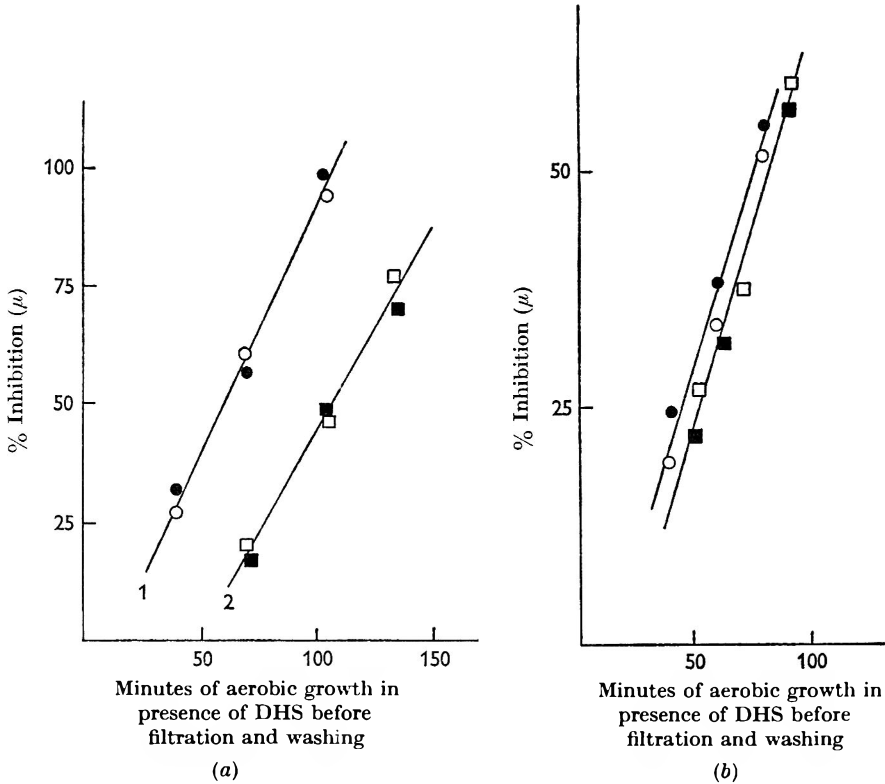

Fig. 5. Percentage inhibition of aerobic and anaerobic growth rates of *E. coli* B as a function of duration of prior aerobic growth in different concentrations of dihydrostreptomycin. (*a*) 20 μg. dihydrostreptomycin sulphate/ml. Two separate experiments: (1) without prior anaerobic growth, (2) addition of drug to aerobic culture after two generations of anaerobic growth. Open symbols = inhibition of aerobic growth, filled symbols = inhibition of anaerobic growth. (*b*) 100 μg. dihydrostreptomycin sulphate/ml., added to aerobic cultures after two generations of anaerobic growth. Two separate experiments represented by circles and squares. Open symbols = inhibition of aerobic growth, filled symbols = inhibition of anaerobic growth.

of the sample for filtration to replacement of the culture in the water bath, was 5–10 min. A typical experiment is depicted in Fig. 4. Dihydrostreptomycin-free controls, which were also subjected to the 'filtration treatment' gave consistent aerobic growth rates for samples filtered at various times, and only one such sample was therefore taken as control for aerobic growth (Fig. 4*c*). Anaerobically, however, the growth rates of the controls sometimes varied with the duration of previous aerobic growth. Therefore samples for filtration were taken from the controls each time a sample was taken from the dihydrostreptomycin-treated cultures (Fig. 4*b*).

In a large number of similar experiments, it was always found that samples withdrawn from the dihydrostreptomycin-treated culture before growth had become completely inhibited, continued to grow, both aerobically and anaerobically, but at rates which were slower in proportion to the duration of the prior aerobic growth in dihydrostreptomycin and to the initial concentration of dihydrostreptomycin in the medium.

As the growth of all filtered samples, both aerobically and anaerobically, appeared to be exponential, it was possible to make a quantitative comparison of inhibition of aerobic and anaerobic growth on the basis of the different growth rates. Figure 5 shows the % inhibition of aerobic and anaerobic growth rates plotted against the duration of the previous aerobic growth in presence of dihydrostreptomycin. These results are from four separate experiments, of the kind illustrated in Fig. 4. The cycle of anaerobic growth before treatment with dihydrostreptomycin, which was introduced in order to abolish the lag period which occurred on changing from aerobic to anaerobic growth, tended to decrease the sensitivity of the organism to dihydrostreptomycin; i.e. it took longer to reach a given degree of inhibition for a given antibiotic concentration. The 'anaerobic lag' gradually returned when cultures were incubated aerobically, and was occasionally apparent in experiments of a duration necessary to obtain a relatively high degree of inhibition with dihydrostreptomycin sulphate 20 μg./ml. In some experiments, therefore, the concentration of dihydrostreptomycin sulphate was increased to 100 μg./ml. so as to obtain approximately 50 % inhibition of growth rate in 30 min. or less (Fig. 5*b*). In all cases, the % inhibition of growth rate, in terms of the appropriate control, was the same for aerobic and anaerobic growth, and appeared to be a function of the duration of treatment with dihydrostreptomycin, and of its initial concentration in the culture medium.

DISCUSSION

Escherichia coli B when growing exponentially under anaerobic conditions was not inhibited by dihydrostreptomycin; this confirmed previous observations with other streptomycin-sensitive organisms. In two types of further experiments cultures of *E. coli* B were exposed to dihydrostreptomycin under conditions which lead to a continued uptake of the antibiotic with an increasing degree of inhibition (Hancock, 1962*b*). When samples of such cultures were removed at different degrees of inhibition and incubated under anaerobic conditions, growth continued at a slower rate, thus indicating that the effects of dihydrostreptomycin on growth, once established, were not dependent on aerobic metabolism. In the first series of experiments it was impossible to compare the degrees of inhibition of aerobic and anaerobic growth produced by a given concentration of dihydrostreptomycin. Aerobically the inhibition was progressive, whereas anaerobically the increase in inhibition was arrested at a value related to the degree of inhibition existing at the time when conditions were changed from aerobic to anaerobic. The findings of Hancock (1962*a*) that anaerobic conditions prevented uptake of streptomycin, whereas under aerobic conditions exponentially growing organisms accumulated streptomycin, offer an explanation of this phenomenon.

The use of the filtration technique in the second series of experiments enabled organisms to be exposed to dihydrostreptomycin for a given time and then further

accumulation prevented by transfer to dihydrostreptomycin-free medium. Such organisms grew exponentially at a slower rate, aerobically and anaerobically. Under these conditions the degree of inhibition in the two states was compared directly and was found to be the same. The lack of dependence of established inhibition of growth rate on aerobic metabolism was found over a wide range of degrees of inhibition and it seems quite clear, therefore, that the need for aerobic metabolism is concerned only with uptake of dihydrostreptomycin. It is possible that the linear relationship between previous duration of growth in the presence of dihydrostreptomycin, and % inhibition of growth rate, reflects a linear relationship between intracellular concentration of dihydrostreptomycin and the growth rate. If it could be shown that the intracellular concentration of dihydrostreptomycin was a linear function of time of exposure, this interpretation would seem reasonable. Although Litwak & Pramer (1957) with Nitella, and Hancock (1962*a*) with several bacterial species showed that uptake of streptomycin was an energy-dependent process, and was affected by a variety of conditions, no adequate quantitative data on rates of uptake are available.

The difference in rate of development of inhibition between *Escherichia coli* B organisms previously exposed to anaerobic conditions, and those not so exposed, is probably due to an effect on the uptake of dihydrostreptomycin; it illustrates again the susceptibility of 'streptomycin' effects to different environmental conditions. The experimental technique described here enabled the uptake of dihydrostreptomycin to be stopped at any desired level, and organisms with different fixed doses of dihydrostreptomycin could then be examined and compared. The inhibited exponential growth of such organisms with subsequent recovery has not been previously described; it is examined more fully in a later paper.

It would seem to be a naïve reflexion that growth-inhibitory substances, in order to manifest their growth-inhibitory effects, will in the majority of cases have to pass through or get inside, at least the outermost layer of the organism. Such processes should be regarded as 'uptake into the organism'. Although with nutrients and metabolites such 'uptake' is no longer thought to be simple diffusion or penetration of a porous surface, it is surprising how little attention has been devoted to the character and mechanism of 'uptake' of antibiotics. Since this is a necessary but presumably quite separate pre-condition for the actual growth-inhibitory effects, a better understanding of the relationship between these two phases of antibiotic action may be required.

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Effects of Dihydrostreptomycin Treatment on the Growth of *Escherichia coli* after Removal of Extracellular Antibiotic

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SUMMARY

The growth characteristics of *Escherichia coli* B cultures treated with dihydrostreptomycin and then freed from extracellular antibiotic before growth had completely stopped, were examined. Growth, measured by extinction, proceeded exponentially, but at slower rates for a time, followed by gradual recovery. The degree of slowing of growth rate was a function of the duration of growth in the presence of a given concentration of dihydrostreptomycin. Comparison of viable colony count data and microscopic observation of such treated cultures showed that the majority of individuals in the populations must grow at the lower rates for two–three generations, after which some organisms cease to multiply and the rest recover. The proportion of organisms in treated populations which eventually ceased to grow was also a function of the duration of treatment. The amount of growth (cell synthesis), which had occurred at the time when onset of recovery became measurable, varied inversely with the % inhibition of growth rate. This suggests that recovery was due to some process not inhibited during the phase of inhibited exponential growth. It is concluded that intracellular dihydrostreptomycin consists of an 'inhibitory fraction' at the sites of inhibition, and a non-inhibitory 'pool' fraction; that the size of the latter varies between different individuals within a population and that transfer from 'pool' to inhibitory sites occurs by a process other than equilibration; e.g. that the factors which govern the uptake into these two phases must be, at least partly, independent. It is suggested that the degree of inhibition of growth rate reflects the extent of combination between antibiotic and inhibition sites at the time when extracellular dihydrostreptomycin is removed and no further uptake into the organisms can occur, and that the complex between dihydrostreptomycin and inhibition sites cannot dissociate to give active antibiotic which could re-enter the 'pool'.

INTRODUCTION

After an extensive survey of the literature for a review on the mode of action of streptomycin (Kogut & Lightbown, 1964*a*), we became impressed with the desirability of dissociating the effects of this antibiotic on microbial growth from those connected with its entry into the organisms. Some aspects of the latter process have been described for a number of organisms by Pramer (1956), Litwak & Pramer (1957), Szybalski & Mashima (1959), Hancock (1962*a*) and Hurwitz & Rosano (1962). In the preceding paper (Kogut, Lightbown & Isaacson, 1965) we described a technique for studying the effects of dihydrostreptomycin on the growth of *Escherichia coli* B, uncomplicated by the continued accumulation of the antibiotic

inside the organisms. The present paper deals with the characteristics of growth inhibition by dihydrostreptomycin under these conditions.

METHODS

The organism, medium, growth conditions, measurement of growth, preparation of inocula, dihydrostreptomycin treatment and procedure for removing extracellular dihydrostreptomycin, were as described by Kogut *et al.* (1965).

Colony counts were made by diluting appropriate samples in sterile growth medium without glucose, and plating on complete growth medium solidified with 1 % (w/v) agar. Pour-plate and, in some experiments, drop counts (Miles & Misra, 1938) were made, each colony count determination being obtained from platings in triplicate from each of two different dilutions. Colonies were counted after incubation at 33–35° for 48–72 hr.

Direct observation of multiplication. For direct observation of the growth and division of bacteria from a culture, two methods of cultivating 20–60 organisms on solid media were used. Method A allowed the initial stage of growth, soon after the removal of extracellular dihydrostreptomycin, to be followed for about three divisions; method B was suitable for the observation of later phases of microcolony formation.

Method A. Samples withdrawn from the culture at the same time as plating for colony counts, were diluted in growth medium + 0·2 % (w/v) agar held at 35–40°, so that a drop which would just fill a Thoma counting chamber of 0·02 mm. depth contained 30–60 organisms. A cover-slip was placed over the drop but not sealed, and the slide immediately placed on the stage of a Cooke, Troughton & Simms binocular microscope which was mounted in a hot box, constructed of Perspex, with a thermostat and heater which allowed the ambient temperature to be held at 33–35°. The organisms were viewed by phase contrast with a × 40 objective and a × 8 ocular, and the position of the organisms in the grid of the Thoma chamber noted. The whole grid was then viewed at about hourly intervals and the numbers and positions of organisms recorded. The success of this method depended on the bacteria retaining their positions in the grid in which they were originally recorded. Usually, the agar set sufficiently for the movement of organisms to cease within 30–60 min. from the time they were placed on the slide. Preparations in which more than a few of the bacteria moved from their original positions after this time had to be discarded. Under these conditions it was possible to count with certainty up to ten organisms derived from individual bacteria at the first viewing, and to estimate up to about sixteen organisms per cluster. This technique allowed the fate of individual bacteria to be observed for about three generations. At later stages, the bacteria became rather crowded and may have exhausted their nutrients. Also, because of the longer generation times in dihydrostreptomycin-treated cultures, incubation beyond this point tended to produce drying of the agar. Postgate, Crumpton & Hunter (1961) have described a similar method for the estimation of viability in a number of organisms. They found that the proportion of bacteria which did not give rise to visible colonies on conventional plate counts was the same as the proportion which did not divide at all during direct microscopic observation for 2–6 hr, according to the organism and the growth temperature; this was approxi-

mately three generation times. They used 'home-brewed tryptic meat medium' and state that the method was not suitable for establishing this relationship in the case of *Escherichia coli* B because these bacteria tended to form long ramifying filaments. Like Bretz (1962) we did not observe such filament formation under our conditions of growth on a mineral salts medium + 0·2 % glucose and incubation at 33–35°.

Method B. In this method, for which we are indebted to Mr M. Young of this Institute, bacteria were cultivated on the surface of agar medium in such a way that they were evenly spread over a fairly large area and could be observed for 24 hr or more. An 'England Finder' slide (Graticules Ltd., London, E.C. 1) was used; this has an etched grid of letters and numbers which can be viewed through a thin agar layer, thus allowing individual bacteria to be repeatedly located. The slide was placed on a levelling table and covered with a thin even layer of 1 % agar nutrient medium by rapidly pipetting on to it 0·5 ml. which had been kept at 80–100°. After this had set (usually in 3–5 min.) it was trimmed with a razor to an area which was a little less than that of a 0·75 in. cover-slip. The slide was then kept in a sterile Petri dish containing moist lint until ready for use. Culture samples, withdrawn at the times of sampling for colony counting, and diluted in growth medium without glucose, were adjusted to concentrations of about 10,000 to 20,000 bacteria/ml. A drop of this suspension (about 0·01 ml.) was placed on the agar square and spread slightly by tilting. When the drop was the right size, and a cover-slip placed carefully on it, the bacteria were evenly distributed. When the drop was too large the placing of the cover-glass tended to crowd the organisms at the edges of the agar square. A seal of liquid paraffin in the space between agar, slide and cover-glass prevented drying of the agar during prolonged incubation. (This means that the bacteria were growing anaerobically most of the time; but we showed in the preceding paper, Kogut *et al.* 1965, that anaerobic and aerobic growth were equally affected after the previous aerobic growth in presence of dihydrostreptomycin.) Two preparations, one for control and one for dihydrostreptomycin-treated cultures, were usually placed on the same England Finder slide and incubated on the microscope stage in a hot box at 33–50°. With this method it was difficult to identify with certainty single bacteria, and to search the area for these in a reasonable period of time (30–60 min.) because of the few organisms originally spread over a comparatively large area, the thickness of the agar, and dust and debris on its surface which could not be avoided. We therefore started observations after a sufficient incubation period to allow for one to two divisions, and scored only units of two or more bacteria. By using phase contrast with a × 20 objective and a × 8 ocular throughout, we recorded the position, by letter and number of the England Finder grid, of 30–60 units of two or more bacteria, at the first search, and then relocated them after periods of incubation and scored their 'growth'. A more rapid method of scanning the slide for groups of fifty or more organisms was suggested by Mr M. Young. This consisted in viewing with a × 20 objective but 95 phase, which gives a picture of lower magnification than ordinary phase and needs less precise focusing. The agar appeared as a dark background, and all debris and objects on its surface as bright spots. Among these, groups of bacteria were easily distinguished by a bluish fluorescent appearance, as well as by their shape.

RESULTS

In the preceding paper (Kogut *et al.* 1965) it was reported that when aerobic cultures of *Escherichia coli* B were taken during exponential growth in the presence of dihydrostreptomycin but before growth had ceased, and treated to remove extracellular antibiotic, they continued to grow, aerobically as well as anaerobically, at apparently exponential though slower rates. It was also shown that the degree of inhibition of growth rate was a function of the duration of previous aerobic growth in presence of dihydrostreptomycin, and of the initial concentration of this antibiotic. Aerobically, this inhibited logarithmic growth appears to proceed for several

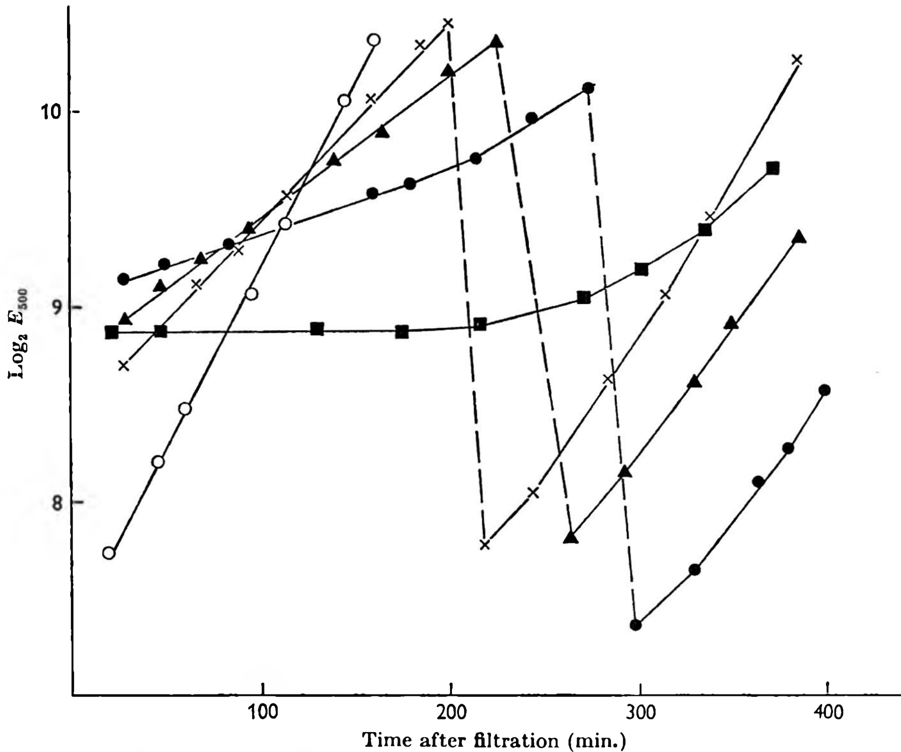

Fig. 1. Aerobic growth of *Escherichia coli* B after treatment with dihydrostreptomycin. Aerobic growth in antibiotic-free medium after various periods of aerobic growth in the presence of dihydrostreptomycin sulphate, 20 μg./ml., and subsequent removal of extracellular dihydrostreptomycin by filtration and washing. ○—○ control culture; dihydrostreptomycin-treated cultures: ×—× filtered after 65 min.; ▲—▲ filtered after 85 min.: ●—● filtered after 105 min.: ■—■ filtered after 125 min. Dashed lines indicate dilution of cultures with fresh medium to allow for continued growth, showing recovery of growth rates.

hours, after which time the growth rates increase and gradually return to those of untreated controls. Figure 1 depicts a representative experiment of this kind, in which three of four samples continued to grow at apparently exponential inhibited rates, and all eventually recovered normal growth rates.

Two features of the inhibited growth of *Escherichia coli* B after treatment which

had allowed intracellular accumulation of dihydrostreptomycin, but in the absence of extracellular antibiotic, seemed relevant to the mode of action of this antibiotic; namely, the apparently exponential nature of inhibited growth during the first few hours and the recovery in growth rates.

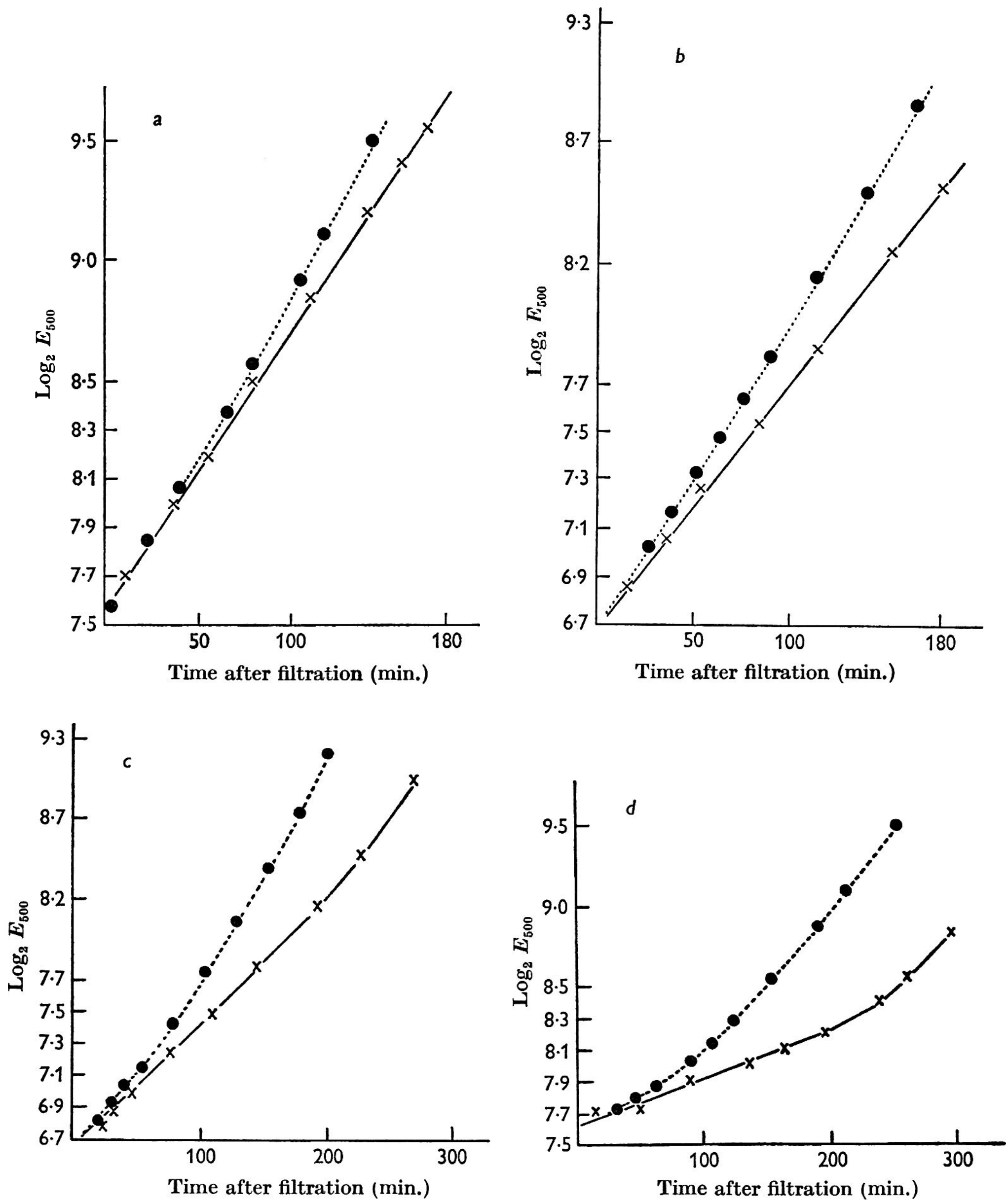

Fig. 2. Observed and calculated growth curves of *Escherichia coli* B after treatment with dihydrostreptomycin sulphate, 20 μg/ml. Calculated growth curves are based on the assumption that % inhibition of growth rate observed equals % of population not growing and the rest growing at uninhibited rates: a = 21 % inhibition of growth rate; b = 34 % inhibition of growth rate; c = 52 % inhibition of growth rate; d = 80 % inhibition of growth rate. × — × = observed growth curves; ● — ● = calculated growth curves.

The apparently exponential nature of inhibited growth. Inspection of the records from many experiments of the above kind showed that in most cases the initial phase of inhibited growth appeared to be truly logarithmic, as far as could be determined over the periods concerned and by the methods available. We considered the

Table 1. *Direct microscopic observation of multiplication of Escherichia coli* B *during periods of exponential inhibited growth and of controls (Method A)*

	Liquid culture		Slide culture		
Expt. no.	Growth rate (μ)	% inhibition (μ)	Initial no. of organisms	Calculated generations on slide (no.)	No. of bacteria not dividing
Controls					
1	0·80	0	80	1·6	4
2	1·00	0	18	3·0	1
3	0·93	0	55	3·26	0
4	1·05	0	65	2·10	3
After treatment with dihydrostreptomycin sulphate, 20 μg./ml. for 70–95 min.					
5	0·44	55	40	3·0	2
6	0·41	58	38	3·0	2
7	0·33	66	30	3·3	4
8	0·58	44	40	3·5	3
9	0·33	66	24	3·0	1
10	0·49	52	37	3·0	1

Table 2. *Proportions of colony-forming units/ml. in logarithmically growing cultures of Escherichia coli* B

Expt. no.	Time after start of expt. (min.)	Growth rate (μ)	E_{500}	Colony-forming units/ml. ($\times 10^{-9}$)	Ratio: colony-forming units/ml./E_{500} ($\times 10^{-9}$)
1	35	0·98	0·311	0·611	1·96
2	135	1·0	0·44	0·86	1·92
3	60	0·96	0·485	0·85	1·75
4	60	1·00	0·383	0·733	1·83
5	60	1·00	0·293	0·58	1·97
6	75	1·03	0·320	0·54	1·69
7	75	1·06	0·345	0·766	2·22
8	0	1·07	0·150	0·305	2·01
	150	1·07	0·966	1·82	1·89
9	0	1·08	0·156	0·267	1·75
	90	1·08	0·597	1·06	1·77

Mean value for colony-forming units/ml./E_{500} = $1·98 \times 10^9$.

possibility that such apparently exponential growth curves might be obtained if a proportion of the population were not growing at all, and the rest were growing at uninhibited rates. Figure 2 compares growth curves calculated on the assumption that x% of the bacteria were not growing at all and the remainder were growing

at uninhibited rates, with those curves actually observed in four experiments, where growth rates were inhibited by x %. It is clear in Fig. 2 that for inhibition of growth rates by more than 20 %, the two sets of growth curves are easily distinguishable. Figure 2 also shows that for each observed growth curve, if x % of the bacteria in the population had not been growing at all, then the rest could not have been growing at uninhibited rates; and if the % of non-growing bacteria was smaller

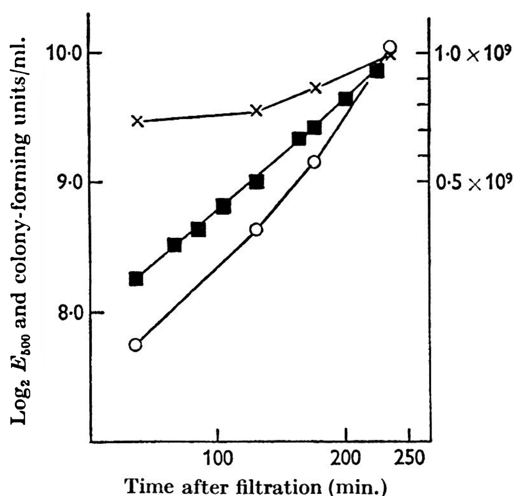

Fig. 3

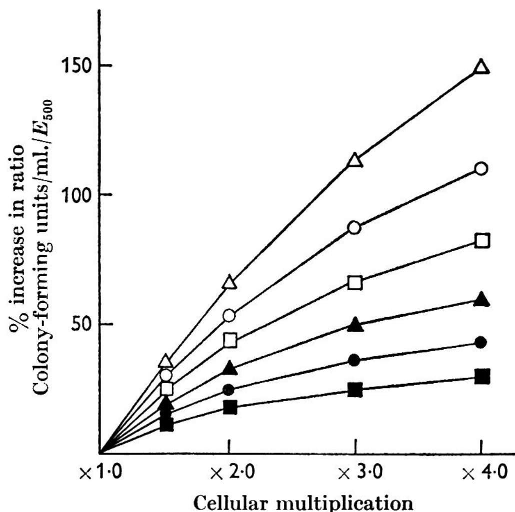

Fig. 4

Fig. 3. Relation between E_{500} and colony counts after treatment of *Escherichia coli* B with dihydrostreptomycin. Effect of aerobic growth for 75 min. in the presence of dihydrostreptomycin, 20 μg/ml., on subsequent increase in extinction, colony-forming units/ml. and ratio of colony-forming units/ml. to E_{500}, after removal of extracellular dihydrostreptomycin. ■—■ = $\log_2 E_{500}$; ○—○ = $\log_2$colony-forming units/ml. (left-hand scale). ×—× = colony-forming units/ml./E_{500} (right-hand scale).

Fig. 4. Relation between colony counts and extinction during growth of populations containing non-dividing bacteria. Percentage increase in the ratio of colony-forming units/ml. to E_{500} as a function of cellular multiplication, calculated for populations assumed to contain various proportions of bacteria which are not multiplying. Proportions of populations assumed to be not growing (%): ■—■ = 30; ●—● = 40; ▲—▲ = 50; □—□ = 60; ○—○ = 70; △—△ = 80.

than x %, the growth rates of the remainder would have to be even slower. In other words, for any population of bacteria containing only those growing normally or not at all, to yield growth curves of the kind observed, the percentage of 'dead' bacteria would have to be larger than the % inhibition of growth rates.

Whether such a situation existed could be tested directly by observing the fate of individual bacteria during periods corresponding to the phase of apparently exponential, but slower growth in liquid culture. With the method A, outlined in Methods, the number of bacteria which had not divided at all, could be evaluated. Table 1 shows that the numbers of bacteria (out of those originally placed on the slides) which had not divided at all in about three generation times were similar in the dihydrostreptomycin-treated cultures and in controls.

Examination of the proportion of bacteria able to give visible colonies. A further

test for the possibility that a certain percentage of the population were not multiplying at all, would be provided by examination of the proportion of bacteria able to give visible colonies. In untreated, exponentially-growing cultures of *Escherichia coli* the colony count increased in parallel with other measurements of growth,

Table 3. *Relation of colony counts and extinction measurements in growing cultures of Escherichia coli* B *after treatment with dihydrostreptomycin sulphate,* 20 *μg./ml*

Observed changes in ratio of colony-forming units/ml./E_{500} with time after treatment with dihydrostreptomycin, compared with calculated changes. Calculations are based on the assumption that % decrease in ratio of colony-forming units/ml./E_{500} from controls = % of population not multiplying, and the changes in ratio of colony-forming units/ml./E_{500} during growth shown in Fig. 4.

Expt. no.	1	2	3	4
Growth in dihydrostreptomycin (min.)	75	75	80	105
Growth rates (μ) after filtration	0·495	0·495	0·438	0·186
Inhibition of growth rates compared to controls (%)	52	52	58	83
Observed changes during growth after filtration				
Sample 1				
T1 time after filtration (min.)	35	35	60	0
E1 E_{500}	0·308	0·308	0·401	0·381
C1 colony-forming units/ml. ($\times 10^{-9}$)	0·229	0·229	0·465	0·144
CE1 colony-forming units/ml./E_{500} ($\times 10^{-9}$)	0·705	0·705	1·160	0·378
Sample 2				
T2 time after T1 (min.)	95	140	75	115
E2 E_{500}	0·520	0·682	0·570	0·471
C2 colony-forming units/ml. ($\times 10^{-9}$)	0·400	0·580	0·730	0·168
CE2 colony-forming units/ml./E_{500} ($\times 10^{-9}$)	0·770	0·850	1·280	0·356
Calculations				
(*a*) $\frac{\text{CE0*} - \text{CE1}}{\text{CE0}} \times 100$ = % decrease in CE1 from controls	62·6	62·6	38·6	80·0
(*b*) C2/C1 = cellular multiplications	1·748	2·540	1·570	1·167
(*c*) $\frac{\text{CE2} - \text{CE1}}{\text{CE1}} \times 100$ = % change in ratio colony-forming units/ml./E_{500} between S1 and S2	+9·2	+20·6	+10·3	−6·2
(*d*) $\frac{\text{'E' CE2} - \text{CE1}}{\text{CE1}} \times 100$ = expected % change in colony-forming units/ml./E_{500} calculated from lines (*a*) and (*b*) and Fig. 4	+38·6	+64·5	+17·5	+19
(*e*) 'E' CE2 = expected ratio of colony-forming units/ml./E_{500} calculated from observed CE1 and line (*d*) ($\times 10^{-9}$)	0·977	1·160	1·363	0·450

* CE0 = colony-forming units/ml./E_{500} for controls, from Table 2 = $1 \cdot 9 \times 10^{9}$.

e.g. extinction (E_{500}) and the ratio of colony count to E_{500}, which is thus a measurement of the proportion of bacteria in the culture able to give visible colonies, was found to be constant (see Table 2). If, after treatment with dihydrostreptomycin, and removal of extracellular antibiotic, some bacteria were unable to multiply at all, and others were multiplying at normal, or even at inhibited rates, then the ratios of colony counts (colony-forming units/ml.)/E_{500} of such cultures should be

increasing with time, at rates determined by the proportions of non-multiplying bacteria and the growth rates of the remainder. Figure 3 shows the rate of change of E_{500} and colony-forming units/ml. (both plotted as $\log_2$) and of the ratio of colony-forming units/ml./E_{500} of a culture filtered after aerobic growth for 75 min. in the presence of 20 μg. dihydrostreptomycin sulphate/ml. when the apparently logarithmic growth rate was inhibited by approximately 50 %. Although the ratio of colony-forming units/ml./E_{500} was much lower than the mean control value (by about 60 %) it remained more or less constant during most of the phase of logarithmic but inhibited growth.

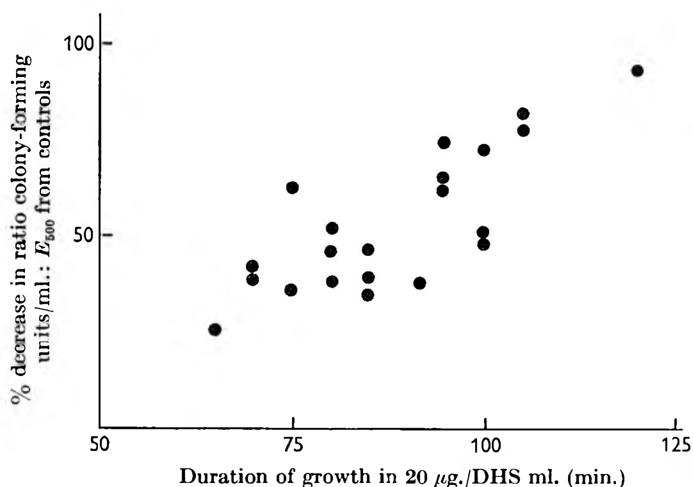

Fig. 5. Loss of colony-forming ability of *Escherichia coli* B as a function of dihydrostreptomycin (DHS) treatment. Percentage decrease in ratio of colony-forming units/ml. to E_{500} from control values, plotted against duration of previous growth in the presence of dihydrostreptomycin sulphate, 20 μg./ml.

It can be calculated that if x % of the bacteria in a population were not multiplying, then the ratio of colony-forming units/ml./E_{500} should increase during growth as illustrated in Fig. 4. With the use of this graph, and assuming that % decrease in colony-forming units/ml./E_{500} from controls = % of population not multiplying, one can calculate the expected increase in colony-forming units/ml./E_{500} for any interval of growth, from the experimental data on growth rates and colony-forming units/ml./E_{500}. Table 3 compares these calculated changes in the ratio of colony-forming units/ml./E_{500} with those actually recorded in a number of experiments. It is clear that the majority of bacteria in these populations must have been multiplying throughout the periods of 'logarithmic but inhibited growth' when the samples were taken. However, when the extent of decrease in colony-forming units/ml./E_{500} (as % of control values) after treatment with dihydrostreptomycin was determined in many experiments, it was found to be a function of the duration of previous aerobic growth in the presence of the antibiotic (as shown in Fig. 5) in the same way as was % inhibition of growth rates.

There was thus an apparent paradox in the behaviour of such dihydrostreptomycin-treated cultures. On the one hand growth of liquid cultures (measured by E_{500}) direct microscopic observation of individual organisms for three generation

times, and the constancy of the ratio colony-forming units/ml./E_{500} during this period, suggested that nearly all the bacteria in such cultures were initially able to multiply. On the other hand experiments which measured the proportion of bacteria able to give visible colonies after prolonged incubation on solid media showed these to be reduced in such dihydrostreptomycin-treated cultures.

These discrepancies might have arisen from differences in the methods involved. Thus some of the bacteria in the antibiotic-treated cultures (their number varying with the duration of treatment) might have been damaged in such a way that some factor involved in the colony count technique, but not in the slide or liquid culture, rendered them unable to divide. Such a situation was described for phenol-treated *Escherichia coli*, including strain B, by Harris & Whitefield (1963). Two such possible factors, namely, altered temperature sensitivity and adherence to glassware, as a result of dihydrostreptomycin-treatment, were considered by us. By using the Miles & Misra drop technique, to avoid contact of bacteria with molten agar, portions of the final dilutions for plating were incubated, for 10 min. each, at room temperature (18°), 40, 45, 48 and 50°. At incubation temperatures of 48° and 50°, all cultures showed a decrease in colony count, but in the dihydrostreptomycin-treated cultures this was more marked than in the controls. After incubation at 40° and 45°, controls and dihydrostreptomycin-treated bacteria behaved similarly; all showed small and variable decreases in colony counts as compared to those incubated at room temperature, but this did not affect significantly the loss in proportion of colony-forming units/ml./E_{500} following antibiotic treatment. In general, and with 10 min. of incubation at room temperature, the loss in proportion of colony-forming units after dihydrostreptomycin treatment appeared slightly larger with the Miles & Misra drop technique than in pour plates (of the order of 10–15 %). In the pour-plate method, the agar was kept in a water bath at 41° before pouring.

If increased adherence to glass were to account for the apparent reduction in viable units, then the percentage loss would increase with the number of serial dilutions used. In the Miles & Misra drop technique the aim is to count about 30 colonies from drops of 0·02 ml., whilst in the plate counts dilutions to about 100 colony-forming units/ml. are used. The latter method, therefore, involves a larger number of serial dilutions, and should give the greater % decrease in colony-forming units/ml./E_{500}. This was not the case.

The possibility that an increased tendency to clump could account for the apparent decrease in colony-forming units/ml./E_{500} with dihydrostreptomycin-treated bacteria, was considered to be unlikely; only occasionally slight clumping was observed in direct microscopic examination and incorporation of 'Lissapol' (Norris & Powell, 1961) into the diluents did not affect the results of plate counts.

A different interpretation of the discrepancy between logarithmic growth in liquid cultures at inhibited rates, coupled with the ability of all bacteria in the cultures to divide for a time, and the inability of a proportion of these bacteria to give visible colonies in conventional plate counts, may be suggested. This is that at some time, corresponding to the end of the period of inhibited logarithmic growth, a proportion (varying in size with the duration of antibiotic treatment) of the bacteria in such populations ceases to divide.

Direct microscopic examination of individual bacteria during prolonged incubation

on nutrient agar. Method B (see Methods) allowed continuous or intermittent observation of several clones over many generations, from the first or second division to the stage of visible colony formation. (With this method B we did not score single bacteria because these could not be identified with sufficient certainty, but started observations on bacteria which had undergone at least one division.) It was readily established with this technique that the decrease in colony-forming units/ml./E_{500} in the dihydrostreptomycin-treated cultures was not due to arrest of growth at a late, micro-colony stage: once a group of 8–10 bacteria had been located

Table 4. *Division and growth of bacteria from dihydrostreptomycin-treated cultures of Escherichia coli* B *during inhibited logarithmic growth and from control cultures, observed microscopically on solid medium (Method B)*

Expt. no.	1	2	3
Growth in dihydrostreptomycin sulphate, 20 μg./ml. (min.)	90	60	65
Growth rates (μ)	0·110	0·540	0·434
% inhibition (μ)	89	49	53·6
% decrease in ratio colony-forming units/ml./E_{500} from control	86·8	31·4	36·0
Time of sampling after filtration (min.)	90	70	70
Incubation temperature of slide	33–35°	32–35°	30–35°

	Control	DHS-treated	Control	DHS-treated	Control	DHS-treated
Calculated generations on slide at first observations	2+	0·5	2–2·75	2·4	1·5–3·5	1·4–3·4
Growth stage (bacteria/group) at first observations	4–20	1–8	2–8	2–20	2–8	2–20
Nos. first judged 'positive' as bacteria having divided	54	26	29	40	28	17
Nos. first judged 'doubtful'	8	27	2	7	4	5
Nos. later confirmed as colonies	62	16	28	34	27	11
Nos. later judged 'not grown'	1	29	2	11	4	11
Nos. lost	0	8	1	2	1	0
% of those first recorded, later not grown	0	70	10	27·5	16	50
% of those first judged 'positive' later not grown or lost	0	38·5	3·5	15	3	35

during the first few hours of incubation on the slide, it always continued to grow and divide; occasionally some (10–20 %) of the clones in the treated cultures appeared somewhat retarded, but they eventually reached the same colony size (diameter) as did those of controls. This left us with the question about what happens during the first few generation times after the removal of extracellular antibiotic. We have shown that with method A one cannot distinguish the dihydrostreptomycin-treated from control cultures on the basis of % of bacteria which undergo no division during three generation times. With method B, however, there was a difference between control and treated cultures. In the former, bacteria which had divided at least once could be recognized with almost complete certainty, and practically all the organisms scored initially as two or more bacteria continued to grow and divide. In the dihydrostreptomycin-treated cultures it was much more difficult to recognize such small groups of bacteria; a much larger proportion of those recorded initially

(particularly of those considered 'doubtful') later could not be recognized or remained unchanged. Table 4 shows a number of such observations on control and dihydrostreptomycin-treated cultures. Recorded are the approximate number of generation times of incubation on the slide at the first observation (calculated from the growth rates in liquid culture) and the growth stage actually reached at the first observation (in numbers of bacteria per group). The wide spread in the latter values can be partly accounted for by the length of time it took to search the slide at this stage (1–3 hr) and partly by the distribution of division times and growth rates within populations. The groups of organisms first recorded are subdivided into those 'judged positively as bacteria having divided' and those classified as 'doubtful' (these latter were usually the smaller groups of bacteria, as mentioned above). In dihydrostreptomycin-treated cultures, none of those first judged as 'doubtful' were later found to have grown, whilst in the controls some of these did later give colonies (expt. 1). Of those first judged as 'positive' in the control cultures, almost all grew to colonies, whereas in the treated cultures a proportion was later found to be of doubtful appearance, unchanged in size, or could no longer be recognized. These observations indicate that in the dihydrostreptomycin-treated cultures, a proportion of bacteria did not divide more than once or twice and that such bacteria may change in appearance, or in some cases lyse, so that not all of these are recorded. Postgate *et al.* (1961) reported that populations of *Aerobacter aerogenes* of intermediate viability (20–70 %) contained a proportion of organisms which appeared to attempt multiplication but failed, and were seen as 'drum-stick or spherical bodies of low contrast and not easily recognized'. Because of this suggestion of altered appearance, the values for 'proportion of bacteria which stopped growing' in Table 4, must be minimal, since one cannot estimate the number of those which might have ceased to divide and changed their appearance by the time the first observations were made.

The recovery in growth rates after inhibited exponential growth of Escherichia coli B *in the absence of extracellular antibiotic*

This second feature of growth of dihydrostreptomycin-treated cultures of *Escherichia coli* B in the absence of extracellular antibiotic, the recovery of growth rates, is illustrated in Fig. 1 and occurred in all cases except where growth was completely inhibited. To observe the whole of this recovery phase and to determine whether the growth rates actually returned to normal, cultures had to be diluted with fresh medium at times when it was judged from extinction measurements that they were nearing exhaustion of nutrients. Under these conditions they returned to normal or very nearly normal growth rates.

Such growth curves showing apparent recovery, at first suggested outgrowth of streptomycin-resistant, or of uninhibited organisms present in the culture at the time of filtration. Duplicate platings for colony counts on the ordinary agar medium and on agar medium + dihydrostreptomycin sulphate 20 μg./ml. were made, on controls and at various times after addition of dihydrostreptomycin. Table 5 shows that in control cultures such resistant organisms numbered about $1–3/10^8$ colony-forming units. In the treated cultures, the highest proportion of resistants found was $20/10^8$ colony-forming units, but there was no consistent accumulation of resistant organisms over total viable organisms with duration of growth (difference

Table 5. *Occurrence and growth of dihydrostreptomycin-resistant bacteria in controls and dihydrostreptomycin-treated cultures of Escherichia coli* B

Expt. no.	1	2	3	4
	Controls			
S1, 0 min.				
Colony-forming units/ml. ($\times 10^{9}$)	0·305	0·243	0·267	
Resistant colony-forming units/ml.	5	7·5	2·5	
Resistants/10^{-8} colony-forming units	1·64	3·0	0·95	
S2				
Time after S1 (min.)	150	140	125	
Colony-forming units/ml. ($\times 10^{-9}$)	1·82	2·1	1·06	
Resistant colony-forming units/ml.	40	30	11	
Resistants/10^{-8} colony-forming units	2·2	1·4	1·0	
Increase in colony-forming units/ml. (S2/S1)	5·95	9·0	4·0	
Increase in resistants/ml. (S2/S1)	8·0	4·0	4·3	
	Dihydrostreptomycin-treated cultures			
S1				
Time after dihydrostreptomycin (min.)	105	240	105	200
Colony-forming units/ml. ($\times 10^{-9}$)	0·144	0·06	0·105	0·53
Resistant colony-forming units/ml.	3·0	12	4·0	5·0
Resistants/10^{-8} colony-forming units	2·1	20	3·8	0·94
S2				
Time after dihydrostreptomycin (min.)	470	470	485	360
Colony-forming units/ml. ($\times 10^{-9}$)	1·8	0·65	0·99	1·95
Resistant colony-forming units/ml.	280	10	40	45
Resistants/10^{-8} colony-forming units	15·5	1·5	4·0	2·3
Increase in colony-forming units/ml. (S2/S1)	12·5	10·8	9·4	3·68
Increase in resistant colony-forming units/ml. (S2/S1)	93	0·83	10·0	9·0

Table 6. *Relation between colony counts in recovery phase and during period of inhibited expontential growth*

Calculation of % of colony-forming units present during inhibited exponential growth phase which could account for colony-forming units present in recovery phase, had they multiplied at uninhibited rates throughout (see text).

Expt. no.	1	2	3	4
S1				
Time after dihydrostreptomycin (min.)	105	105	200	140
E_{500}	0·381	0·310	0·465	0·401
Colony-forming units/ml. ($\times 10^{-9}$)	0·144	0·105	0·53	0·465
S2				
Time after dihydrostreptomycin (min.)	470	465	360	310
E_{500}	1·286	0·774	1·282	1·018
Colony-forming units/ml. ($\times 10^{-9}$)	1·8	0·99	1·95	1·43
S2-S1				
Time (hr)	6·08	6·33	2·66	2·83
Generations observed	3·64	3·24	1·88	1·62
Generations calculated if $\mu = 1{\cdot}0$	6·08	6·33	2·66	2·83
Calculated colony-forming units/ml. at S1, if all those counted at S2 had arisen at $\mu = 1{\cdot}0$ ($\times 10^{-9}$)	0·027	0·012	0·308	0·201
As % of observed colony-forming units/ml. at S1	18·5	11·7	58	43

between S 1 and S 2). Only in one case (expt. 1) was there a greater increase in the number of resistant organisms/ml. (93-fold) than in the total colony count/ml. (12·5-fold) during 400 min. of growth. In this experiment, the last count, at 470 min. after adding dihydrostreptomycin, was well within the recovery phase of the growth curve, but it can be easily seen that 280 resistant organisms/ml. would have been quite undetectable by extinction measurements (E_{500} 1·0 = $1·9 \times 10^9$ colony-forming units/ml.). It is obvious, therefore, that the apparent recovery in growth rates

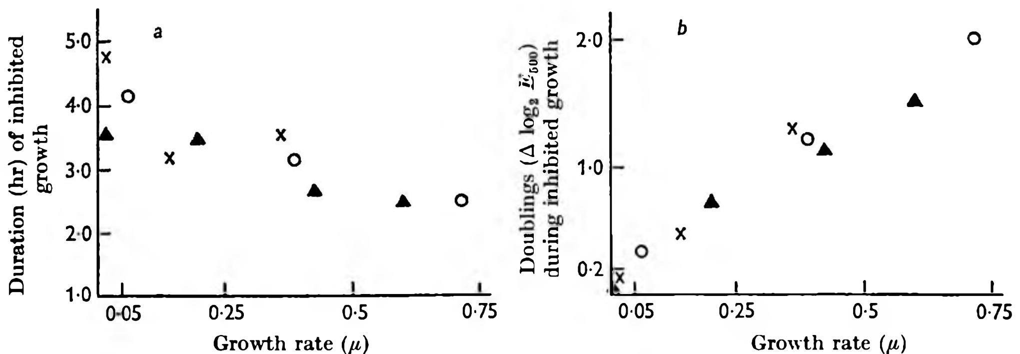

Fig. 6. Duration and extent of inhibited exponential growth of *Escherichia coli* B before onset of recovery as a function of growth rates. (*a*) Duration of inhibited exponential growth before onset of recovery. (*b*) Doublings, measured as $\Delta \log_2 E_{500}$, before onset of recovery. Different symbols are from different experiments.

which usually followed inhibited logarithmic growth after treatment with dihydrostreptomycin and removal of extracellular antibiotic, could not have been due to outgrowth of pre-existing organisms resistant to dihydrostreptomycin 20 μg./ml. or to sudden bursts of mutations giving resistant organisms in sufficient numbers.

Regarding the possibility that continued uninhibited growth of some bacteria might account for the observed recovery phase, it has already been shown by consideration of Fig. 2 that if the observed growth curves were contributed in part by growth of individual organisms at uninhibited rates, then the proportions of non-multiplying bacteria would have to be larger than those found. It can also be calculated from experiments in which colony-forming units/ml. were determined in the recovery phase as well as during the phase of inhibited logarithmic growth (Table 6) that if all those colony-forming units counted in the recovery phase had arisen at uninhibited rates ($\mu = 1$) then they could have constituted only a fraction of the bacteria counted during the logarithmic phase of inhibited growth as able to give visible colonies.

It would appear therefore, that the observed increases in growth rates, whether measured by extinction or colony-forming units (Fig. 3) must represent genuine recovery of at least a substantial portion of the treated populations. It would seem reasonable to assume that such recovery from inhibition would set in after a certain amount of cell synthesis had occurred; in other words that it would depend on the number of cell-multiplications, so that the inhibited, or inhibitory, factors were diluted out by multiplication. In such a case one would expect the onset of recovery to appear after a given number of doublings in bacterial mass or numbers, irrespec-

tive of the growth rates, and hence to occur later on the time scale in the more inhibited cultures. Although the time of onset of recovery cannot be evaluated with precision, inspection of Fig. 1 shows that it was more or less the same in all the samples, whilst total cell synthesis (difference in $\log_2 E_{500}$ between filtration and onset of recovery) which had occurred at this time varied in the different samples. In Fig. 6 the duration of inhibited logarithmic growth before onset of recovery (Fig. 6*a*) and the total cell-synthesis during this period (expressed as doublings) (Fig. 6*b*) are plotted against the growth rates for several experiments. It can be seen in Fig. 6*a* that for a 20-fold change in growth rate, the estimated time of onset of recovery varied only about 2-fold. The doublings in cell mass, synthesized during inhibited growth, and before the onset of recovery, however, varied directly with the growth rate. Such a situation suggests that recovery must be a consequence of some process which is not inhibited, during the time that exponential growth is inhibited, by the treatment with dihydrostreptomycin.

DISCUSSION

Most of the literature on the mode of action of streptomycin deals with experiments where the uptake of the antibiotic by the cells is continuous, so that intracellular concentrations are not constant but increasing; hence the inhibition of growth appears to be cumulative. Under these conditions, factors which influence the intracellular accumulation of antibiotic cannot be distinguished from those which affect its intracellular mode of action, and many of the diverse effects of environmental factors on the antibiotic action of streptomycin and dihydrostreptomycin reported in the literature, might have been due to effects on their uptake into the cells, and hence their intracellular concentrations (Kogut & Lightbown, 1964*a*).

The present and earlier studies (Kogut & Lightbown, 1963, 1964*b*) have shown that after treatment of *Escherichia coli* B with low concentrations of dihydrostreptomycin under conditions where uptake and intracellular accumulation are known to occur (Hancock, 1962*a*; Hurwitz & Rosano, 1962) and subsequent removal of extracellular antibiotic at various times, the cultures continue to grow at slower but exponential rates for periods of several hours, after which the growth rates gradually return to normal. Dubin & Davis (1962) used a similar technique to study what they described as 'streptomycin-triggered' effects. They apparently assumed that filtration and washing removed the intracellular as well as the extracellular antibiotic. We, on the other hand, assume, supported by the data of Hancock (1962*a*), that after such filtration and washing treatments, the cultures continue to grow in the presence of more or less constant amounts of intracellular antibiotic. However, cultures are populations of individuals which vary, within certain limits, in physiological properties, so that individual values are distributed around a mean. The parameters which are usually measured, such as growth rates, are mean values, and the intracellular concentrations of dihydrostreptomycin in the individual bacteria of such cultures should also be distributed around a mean. The extent of inhibition of exponential growth in treated populations was found to be a function of the duration of previous aerobic growth in a given external concentration (μg./ml.) of dihydrostreptomycin. It seems reasonable to assume that it is a function of the mean intracellular concentration of the drug in the individuals of

the population. However, although such populations grow exponentially but at slower rates for several hours, colony count data and direct microscopic observations show that later some of the bacteria cease to multiply, whilst others multiply at accelerating rates and eventually return to normal growth rates. The proportion of bacteria in these two fractions (estimated from measurements of colony-forming units/ml./E_{500}) was almost constant during the exponential phase of inhibited growth, and was a function of the duration of previous growth in a given concentration of dihydrostreptomycin. Hurwitz, Landau & Doppel (1962) claimed that the loss of ability to form macro-colonies after exposure of *Escherichia coli* to streptomycin closely paralleled the loss of ability to divide at all. They correlated the % of bacteria from streptomycin-treated cultures which did not give visible colonies in colony counts with the % of bacteria which did not undergo division when observed microscopically during incubation for 90 min. on slides covered with nutrient agar. They treated cultures in nutrient broth with 1 μg. streptomycin/ml. for 1, 17·5 and 35 min. Since they gave no indication of how this treatment affected the growth rates of their populations, and since there were no data for untreated controls, it is difficult to evaluate whether incubation for 90 min. on the slides was sufficient for any division to have occurred. From our experience it would seem that in their third experiment, where they state that 99 % of the population was unable to give macro-colonies, the growth rate must have been similarly inhibited, so that the mean generation time would have been increased by a factor of about 10, and no division would be expected to occur during 90 min. of incubation.

It seems to us of importance, at this point, to examine the significance and interpretation of 'viable count' measurements in relation to antibiotics. To state the obvious, the technique of 'viable', or colony counting, measures the number of organisms, or units, in a measured sample of a population which can grow and divide sufficiently to give visible colonies. When a growth-inhibitory substance, such as dihydrostreptomycin, or streptomycin is added to a population, and samples removed for viable counting, then provided that a sufficient dose were taken up rapidly by all the individuals, and this was not removed from the bacteria by the dilutions and washing involved in the technique, the viable count should immediately fall to zero. This is not usually the case, mainly because the bacteria do not accumulate growth-inhibitory concentrations of the drug instantaneously. In such cases, e.g. with such growth-inhibitory substances, 'viable counts' then measure the number of units in the population which do not contain growth-inhibitory concentrations. The use of the term 'bactericidal' when referring to the effects of antibiotics which are known to be growth-inhibitory under the experimental conditions used, seems meaningless, unless one could ensure that all, including the intracellularly held, antibiotic were removed from the organisms prior to plating; or its use must be merely operational, denoting the irreversibility of binding at the inhibitory sites. In the case of streptomycin, available evidence (Hancock, 1962*a*, *b*, Spotts, 1962) suggests in fact that it is very tightly bound to some inhibitory sites. The decline in 'viable count', and in our case, the reduction in the ratio of colony-forming units/E_{500}, as a function of the duration of treatment with the antibiotic, must therefore reflect the rate of acquisition and retention of growth-inhibitory antibiotic concentration by individuals in the population. In other words, the ratio of colony-forming units/ml./E_{500} after a given treatment with dihydrostreptomycin

should measure the proportions of bacteria in the treated population whose intracellular concentrations of the drug are insufficient to inhibit growth completely.

It has been suggested that streptomycin (and dihydrostreptomycin) acts by combining with ribosomes, free from messenger RNA, and prevents their functioning in protein synthesis (Erdős & Ullman, 1959; Spotts & Stanier, 1961; Flaks, Cox & White, 1962; Flaks *et al.* 1962; Speyer, Lengyel & Basilio, 1962; Mager, Benedict & Artman, 1962; Davies, 1964). It has also been shown that in normal exponentially growing cultures of bacteria the rate of protein synthesis per (functioning) ribosome is constant, and that changes in growth rates are reflected by changes in ribosome content per cell (Schaechter, Maaløe & Kjeldgaard, 1958). If one assumes that changes in growth rates could be brought about by changes in content of functioning ribosomes, then exponential growth of dihydrostreptomycin-treated populations at rates equal to x % of those of controls could mean that the bacteria were multiplying with x % of their ribosomes functioning. If this were the case, the inhibition of growth rate should be proportional to the degree of saturation of the inhibitory sites. As y % of such populations later cease to grow, and must therefore have intracellular drug concentrations which will eventually inactivate all their ribosomes, it would follow that the total intracellular antibiotic concentrations of these bacteria must be larger than those necessary to saturate the inhibitory sites initially. We thus conclude that intracellular dihydrostreptomycin (and presumably streptomycin) can be in two phases: an inhibitory fraction at the sites of inhibition (presumably the ribosomes) and a non-inhibitory 'pool' fraction from which the drug could be transferred to inhibitory sites when these become available. The sizes of such pools are assumed to differ in different individuals of treated populations. It is implicit in this hypothesis that there cannot be free interchange between 'pool' and inhibitory sites. The statement by Hancock (1962*b*) that 'death of the organisms does not appear to follow inevitably the attainment of a certain intracellular concentration of streptomycin, since at lower streptomycin concentrations the organisms were killed after a smaller amount of streptomycin had been taken up and vice versa' already led us to conjecture that the intracellular antibiotic might be heterogenously distributed, and that the amounts in the different phases might be governed by independent factors. Spotts (1962) showed that intracellular streptomycin accumulated by a streptomycin-dependent strain of *Escherichia coli* consisted of a 'loosely held pool, extractable with hot water, and a firmly-bound fraction, extractable only by hot perchloric acid'. On the other hand, attempts at differential extraction of intracellular ^{14}C-streptomycin by Hancock (1962*b*) gave no clear-cut results. It seems likely that the existence and size of such 'pools' might vary with the organism and conditions of growth (Kogut & Lightbown, 1964*a*).

In our experiments, then, degree of inhibition of growth rates (which are population means) would reflect the degree of saturation of the inhibitory sites at the time of removal of extracellular dihydrostreptomycin, whilst the different percentages of bacteria in the populations which eventually ceased to grow would reflect the different distributions of total intracellular concentrations (and hence pool sizes) of dihydrostreptomycin within these populations. Thus the y % of the treated populations which eventually ceased to grow would be those whose 'pools' were sufficiently large and stable to continue to transfer antibiotic for combination with 'free' ribosomes as these became available. The rest of the populations, whose

growth rates eventually recover, would be those whose 'pools' were exhausted before all ribosomes had been inactivated. The fact that the onset of recovery in growth rates does not depend on a given amount of cell synthesis, but occurs after smaller amounts of synthesis with increasing inhibition of growth rate, would suggest that synthesis of ribosomes is uninhibited in the presence of intracellular dihydrostreptomycin as long as growth inhibition is not complete. Such continued ribosome synthesis could then lead to recovery of growth when all intracellular antibiotic had been bound to existing ribosomes and had become unavailable for further combination with new ribosomes. This view implies that active antibiotic cannot be regenerated from the inhibitory 'ribosome-dihydrostreptomycin complex' and hence cannot re-enter the 'pool'.

Whilst this paper was in preparation, Hurwitz, Doppel & Rosano (1964) reported on the effects of duration of exposure to various concentrations of streptomycin on viability and protein synthesizing ability of *Mycobacterium fortuitum*. Their data on ^{14}C-leucine incorporation, as a measure of protein synthesis, provide a striking parallel to ours on growth, measured by extinction, for the eventual recovery of normal rates after removal of extracellular antibiotic.

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A Biochemical Characterization of Histidine-Dependent Mutants of *Staphylococcus aureus*

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SUMMARY

Over 100 histidine-dependent mutants were isolated from strain 655 of *Staphylococcus aureus*. Paper chromatography was used to differentiate the mutants into classes according to their ability to accumulate Pauly-positive imidazoles. Classes *E* and *G* did not accumulate any detectable Pauly-positive imidazoles. Mutants of class *A* accumulated 5-amino-1-ribosyl-4-imidazolecarboxamide, mutants of class *B* accumulated imidazoleglycerol, and mutants of class *C* accumulated imidazoleacetol. Mutants of class *D* accumulated histidinol and were incapable of utilizing exogenous L-histidinol. A comparison of the accumulations obtained from mutants of *S. aureus* with accumulations obtained from previously characterized mutants of *Salmonella typhimurium* indicates that a similar, if not identical, pathway for the biosynthesis of L-histidine is used by these two species.

INTRODUCTION

Vogel, Davis & Mingioli (1951) isolated L-histidinol from a histidine-dependent mutant of *Escherichia coli* and observed that this compound could support the growth of another histidine-dependent mutant. A chromatographic analysis of imidazoles accumulated by histidine-dependent mutants of *Neurospora crassa* indicated the existence of at least four classes of biotypes (Haas, Mitchell, Ames & Mitchell, 1952). These mutants accumulated Pauly-positive imidazoles which were tentatively identified as imidazoleglycerol (IG), imidazoleacetol (IA) and histidinol (Ames, Mitchell & Mitchell, 1953). Subsequent studies of these mutants revealed that they accumulated the phosphate esters imidazoleglycerol phosphate (IGP), imidazoleacetol phosphate (IAP) and histidinol phosphate (HP) in addition to the unphosphorylated compounds (Ames & Mitchell, 1955). None of the imidazoles accumulated by these histidine-dependent mutants was capable of relieving the histidine requirement of other mutants. Hartman (1956) identified four classes of histidine-dependent mutants of *Salmonella typhimurium*. *A* mutants were distinguished by failure to accumulate Pauly-positive imidazoles. *B* mutants accumulated IG and IGP. *C* mutants accumulated IA, IAP, imidazolelactic acid (ILA) and smaller quantities of IG and IGP. *D* mutants accumulated histidinol, HP, and smaller quantities of IA, IAP, ILA, IG and IGP. Of the imidazoles accumulated, only histidinol and ILA supported the growth of mutants blocked at earlier reactions, while the other imidazoles were considered to be either impermeable to the cell membrane (e.g. phosphate esters) or not actual precursors of L-histidine (e.g. IG and IA). Genetic complementation and quantitative cell-free enzyme analyses

of various *A* mutants showed the presence of at least four different phenotypic classes, designated *E*, *A*, *H* and *F* (Hartman, Loper & Serman, 1960). The genetic studies by these workers indicated the presence of another phenotypic class of mutants, designated *G*, which like the *A* mutants did not accumulate Pauly-positive imidazoles. However, unlike the *A* mutants, the *G* mutants were located adjacent to the *D* locus and thus at a considerable distance from the *E*, *A*, *H* and *F* loci. Smith & Ames (1964) separated the *E* mutants into two distinct classes, *E* and *I*. The newly designated *E* mutants accumulated the Bound Bratton–Marshall compound BBM I, whereas *I* mutants did not accumulate any Bound Bratton–Marshall compounds. A more complete understanding of the pathway used for the biosynthesis of L-histidine has been possible as a result of the studies of specific enzyme activities in cell-free extracts of various histidine-dependent mutants of *S. typhimurium* (Ames & Hartman, 1962; Smith & Ames, 1964).

The present report describes a paper-chromatographic analysis of Pauly-positive imidazoles accumulated by histidine-dependent mutants of *Staphylococcus aureus*, and the degree of similarity of these compounds to those obtained from previously characterized histidine-dependent mutants of *Salmonella typhimurium*. Information about the ability of histidine-dependent mutants of *Staphylococcus aureus* to utilize various imidazoles in place of exogenous L-histidine also is presented. The companion paper (Kloos & Pattee, 1965) describes the genetic analysis of these mutants of *S. aureus*.

METHODS

Media. In addition to commercially available dehydrated media, two defined media were used in this work. Studies involving mutants of *Salmonella typhimurium* were made with the defined medium ('E' broth) of Vogel & Bonner (1956) supplemented with various concentrations of L-histidine (monohydrate; California Corporation for Biochemical Research (Calbiochem), Los Angeles, Calif., U.S.A.). The defined medium (S broth) used for the study of mutants of *Staphylococcus aureus* consisted of the following ingredients: K_2HPO_4, 7 g.; KH_2PO_4, 2 g.; sodium citrate.$5H_2O$, 0·5 g.; $MgSO_4.7H_2O$, 0·1 g., $(NH_4)_2SO_4$, 1 g.; glucose, 4 g.; glycine, 50 mg.; L-glutamic acid, 100 mg.; L-aspartic acid, 90 mg.; L-serine, 30 mg.; L-methionine, 3 mg.; L-cystine, 20 mg.; L-alanine, 60 mg.; L-lysine.HCl, 50 mg.; L-arginine.HCl, 50 mg.; L-threonine, 30 mg.; L-phenylalanine, 40 mg.; L-tryptophan, 10 mg.; L-leucine, 90 mg.; L-isoleucine, 30 mg.; L-proline, 80 mg.; L-valine, 80 mg.; nicotinic acid, 1 mg.; thiamine.HCl, 1 mg.; de-ionized water, 1000 ml. Glucose was added aseptically after sterilization of the defined medium. The concentration of either L-histidine or L-histidinol (dihydrochloride; Mann Research Laboratories, New York, N.Y.) added to S broth was varied according to requirements. Defined medium agar (S agar) was prepared by adding 1·5 % (w/v) Noble agar (Difco) to S broth. All glassware used with the defined media was cleaned in chromic+sulphuric acid mixture and rinsed thoroughly with deionized water before use.

Bacterial strains. The histidine-dependent mutants *hisE-11*, *hisF-41*, *hisA-3*, *hisH-107*, *hisB-456*, *hisC-2*, *hisD-494* and *hisG-46* of *Salmonella typhimurium* were obtained from Dr P. E. Hartman (The Johns Hopkins University); these mutants were described by Hartman *et al.* (1960). All histidine-dependent mutants of

Staphylococcus aureus were obtained from either strain 655 (Pattee & Baldwin, 1962) or a penicillinase-deficient mutant of this strain. All strains of bacteria were maintained at 4° on Brain Heart Infusion (BHI; Difco) agar slopes in screw-capped tubes. The inocula for all experiments were obtained from subcultures of these stocks, previously prepared on BHI agar slopes incubated at 37° for 12 hr and then stored at 4°. These subcultures were replaced at weekly intervals. In all instances, the shaking of cultures was performed with a Burrell Wrist-Action Shaker, (2223 Fifth Avenue, Pittsburgh, Pa, U.S.A.) with the arms mounted over water baths. All growth experiments were conducted at 37°.

Isolation of mutants. Sixteen histidine-dependent mutants were obtained from *Staphylococcus aureus* strain 655 during preliminary studies. The majority of the mutants, however, were derived from a single penicillinase-deficient mutant of strain 655 by means of ultraviolet irradiation and the following modification of the penicillin selection technique of Davis (1949) and Lederberg (1950). A 12 hr BHI agar slope culture of the parent strain was suspended in 5 ml. saline, and 0·1 ml. of this suspension added to each of two tubes containing 5 ml. S broth. The tubes were shaken for 12 hr at a shaker setting of 7. At the end of this period, the cultures were centrifuged and the packed cocci uniformly suspended in 5 ml. saline. The suspensions were mixed in an open flat-bottomed Petri dish (100 mm. diam.) and exposed for 45 sec. and with constant agitation to radiation from a 30-Watt G. E. Germicidal bulb at a distance of 85 cm. Two tubes, each containing 5 ml. S broth, were inoculated with 0·1 ml. samples of the u.v.-irradiated suspension. Both tubes were shaken at a shaker setting of 7, and penicillin G (1 unit/ml.) added after the fifth hour. After further incubation for 26 hr during which shaking was continued, the cultures were centrifuged, the cocci washed once with saline and resuspended in 3 ml. saline. Dilutions of 1/10 and 1/100 of the final coccal suspension were prepared, and 0·1 ml. samples spread over the surfaces of Trypticase Soy Agar (TSA; Baltimore Biological Co.) plates. These plates were incubated for 24 hr, after which the colonies were inoculated by the replica plating technique (Lederberg & Lederberg, 1952) on to TSA and S agar plates. After incubation of the replica plates for 24 hr, those colonies which grew well on TSA but did not do so on S agar were sub-cultured for further study. All isolates were typed with bacteriophages 29, 52, 52 A, 79, 80, 81, 6, 7, 83, 42 B, 47 C, 47, 53, 54, 70, 73, 75, 77 and 44 A of the International Typing Series. These phages and the phage-typing procedure were described by Pattee & Baldwin (1961). The growth response of each isolate to exogenous L-histidine then was determined by spreading about 10^7 cocci of the isolate over the surface of an S agar plate. Several crystals of L-histidine were placed at the centre of the plate, which was incubated for 24 hr and examined for evidence of growth. Strains which did not grow on S agar except in the presence of exogenous L-histidine were maintained for further study.

Designation of mutants. Based upon the nature of their primary accumulations of Pauly-positive imidazoles, the mutants of *Staphylococcus aureus* were placed in classes as follows: *hisA* mutants accumulate 5-amino-1-ribosyl-4-imidazolecarboxamide (AIC-R); *hisB* mutants accumulate imidazoleglycerol (IG); *hisC* mutants accumulate imidazoleacetol (IA); *hisD* mutants accumulate histidinol. The *hisE* and *hisG* mutants did not accumulate Pauly-positive imidazoles, and were differentiated on the basis of their chromosomal position (Kloos & Pattee, 1965).

The mutants have been designated according to the scheme of Hartman *et al.* (1960); for example, mutant *hisAa-83* is the 83rd histidine-dependent mutant isolated, accumulates AIC-R, and possesses a mutant site in the *a* complementation unit of the *A* gene (Kloos & Pattee, 1965).

Methods of growth stimulation. All histidine-dependent mutants of *Staphylococcus aureus* were examined for their ability to utilize exogenous L-histidinol in place of L-histidine by the same methods as used to determine the histidine-dependent nature of the mutants. Mutants *hisAa-11, hisAb-1, hisAb-20, hisD-8* and *hisGb-15* were also tested for their ability to grow on S agar containing either histidinol, IG, IGP, IA, IAP, ILA or HP. These imidazoles were obtained from lyophilized supernatant fluids of cultures of appropriate histidine-dependent mutants of *Salmonella typhimurium* by preparative thin-layer chromatography.

Preparation of cultures for chromatographic analysis. To study the accumulation of Pauly-positive imidazoles by histidine-dependent mutants of *Staphylococcus aureus*, it was necessary to examine several variables in growth conditions. The results of this preliminary work led to adoption of the following procedure. A 12 hr BHI agar slope culture of a mutant was used to inoculate 5 ml. S broth containing 20 μg. L-histidine/ml. The initial coccal concentration of this culture, referred to hereafter as the adaptation culture, was about 1×10^7 cocci/ml. The culture was shaken at a shaker setting of 3 for 12 hr. One-tenth ml. of the adaptation culture was then transferred to 5 ml. S broth containing 2·0 μg. L-histidine or L-histidinol/ml. This culture, referred to hereafter as the de-repression culture, was prepared with L-histidine for the study of the parent strain and *D* mutants, these mutants being incapable of utilizing exogenous L-histidinol. The de-repression cultures were shaken at a shaker setting of 3 for 72 hr, after which the cocci were sedimented by centrifugation and the supernatant fluids lyophilized. The dried residue was then reconstituted in 0·2 ml. de-ionized water and analysed by ascending paper chromatography. Lyophilized supernatant fluids of cultures of *Salmonella typhimurium* were prepared in the manner described above, using 'E' broth supplemented with 20 μg. L-histidine/ml. in the adaptation cultures, and 3·0 μg. L-histidine/ml. in the de-repression cultures.

Chromatography. Pauly-positive imidazoles were identified by ascending paper chromatography. The solvents used for the development of chromatograms were: (*a*) *tert*-butyl alcohol + 50 % formic acid in water (7 + 3 by vol., Ames & Mitchell, 1955); (*b*) *n*-butyl alcohol saturated with 2 N-NH_3 (Block, Durrum & Zweig, 1958); (*c*) *n*-propanol + 0·2 N-NH_3 (3 + 1 by vol., Ames & Mitchell, 1952). Chromatograms were prepared on Whatman 3 MM filter paper, 0·03 ml. of concentrated supernatant fluid being applied as a spot on the origin. Three chromatograms of each mutant were prepared, to permit three solvent systems to be used in development. After each chromatogram was completely dry, it was developed in one of the solvents until the solvent front was 15 cm. from the origin (about 30 min). Each chromatogram then was dried for at least 2 hr at room temperature, after which it was sprayed with diazo sulphanilic acid reagent followed by a 5% (w/v) solution of Na_2CO_3 according to the modified Pauly technique of Ames & Mitchell (1952).

Chemical standards used in the chromatographic analysis were L-histidine, L-histidinol, L-tyrosine, 5-amino-4-imidazolecarboxamide (AIC; Calbiochem),

AIC-R (Calbiochem), imidazoleglycerol, and imidazolelactic acid. The last two imidazoles were provided by Dr P. E. Hartman. In addition to the chemical standards, lyophilized and reconstituted supernatant fluids from cultures of histidine-dependent mutants of *Salmonella typhimurium* were used as sources of known imidazoles.

RESULTS

Isolation of mutants. A total of 85 histidine-dependent mutants were obtained from strain 655 of *Staphylococcus aureus* by the penicillin selection technique. These mutants represented about 0·26 % of the total number of colonies recovered on TSA agar. An additional 75 mutants requiring exogenous purines, pyrimidines or vitamins were also obtained, these nutrients being absent from S broth and S agar.

Growth stimulations. With the exception of the *D* mutants, L-histidinol stimulated all histidine-dependent mutants of *Staphylococcus aureus*. Mutant *hisAaD-63*, which accumulated AIC-R, was not stimulated by L-histidinol. When added to de-repression cultures at concentrations of 10 μg./ml. or greater, L-histidinol inhibited the growth of those mutants of *S. aureus* which were capable of utilizing this compound. Growth of these mutants on S agar also was inhibited by L-histidinol, as evidenced by the zones of growth inhibition surrounding the area where crystalline L-histidinol was deposited. All mutants of *S. aureus* tested (*hisAa-11*, *hisAb-1*, *hisAb-20*, *hisD-8*, *hisGb-15*) did not grow on S agar in the presence of either IG, IGP, IA, IAP, ILA or HP obtained from mutants of *Salmonella typhimurium*. With the exception of mutant *hisD-8*, these mutants were stimulated by histidinol obtained from mutant *hisD-494* of *S. typhimurium*.

Accumulations. The solvents and spray reagents which were used in the final characterization of imidazoles, and the R_F values and colours of these imidazoles are given in Table 1. The parent strain and all the histidine-dependent mutants of *Staphylococcus aureus* accumulated a compound which was indistinguishable from L-tyrosine, and which interfered with the detection of IG. These two compounds were most successfully resolved by means of the *n*-butanol + ammonia solvent system. The colour reaction of L-tyrosine also was weak when this solvent system was used. None of the solvent systems used was capable of adequately resolving IA from histidinol since both of these compounds showed considerable trailing and possessed similar R_F values. However, a colour distinction between IA (red) and histidinol (yellow) was obtained with a sulphanilamide spray reagent (Block *et al.* 1958). The phosphorylated imidazoles IAP, IGP and HP could not be identified in supernatant fluids of cultures of *S. aureus* because of their low R_F values, which placed them in an area of non-specific material and pigments near the origin.

The imidazoles accumulated by each histidine-dependent mutant of *Staphylococcus aureus* are shown in Table 2. Mutants *his-26*, *his-28*, *his-53*, *his-57*, *his-67*, *his-86*, *his-92*, *his-97* and *his-102* grew poorly, did not accumulate detectable quantities of Pauly-positive imidazoles and were excluded from the chromatographic analysis. In addition to the primary accumulations, secondary accumulations were detected in supernatant fluids of cultures of those mutants which exhibited good growth. Although many of the *D* mutants may have accumulated small amounts of IA, this compound would not have been detectable in the presence of histidinol, the primary accumulation product of all *D* mutants. The parent strain normally accumulated small amounts of IG and histidine, although only small

Table 1. *Characterization by ascending paper chromatography of the Pauly-positive imidazoles accumulated by histidine-dependent mutants of Staphylococcus aureus and Salmonella typhimurium*

Compounds listed in the table are as follows: AIC = 5-amino-4-imidazolecarboxamide; AIC-R = 5-amino-1-ribosyl-4-imidazolecarboxamide; IG = imidazoleglycerol; IA = imidazoleacetol; ILA = imidazolelactic acid; L-histidinol; L-histidine; L-tyrosine.

Colour 1 refers to the colour prodnced after the chromatogram was sprayed with diazo sulphanilic acid reagent. The failure to produce any colour after the nse of this reagent is indicated by the symbol –. Colour 2 refers to the colour produced after spraying the chromatogram with diazo sulphanilic acid reagent followed by 5 % (w/v) Na_2CO_3.

	Solvent								
	n-Bntanol + 50 % formic acid in water (7 + 3 by vol.)			n-Propanol + 0·2 N-NH_3 in water (3 + 1 by vol.)			n-Butanol saturated with 2 N-NH_3 in water		
Compound	Colour 1	Colour 2	R_F	Colour 1	Colour 2	R_F	Colour 1	Colour 2	R_F
AIC	Pink	Blue	0·28	Pink-orange	Blue	0·40	Pink-orange	Blue	0·25
AIC-R	Pink	Red-violet	0·20	Pink-orange	Grey-blue	0·28	Pink-orange	Grey-blue	0·09
IG	–	Olive	0·37	–	Brown	0·42	–	Brown	0·18
IA	–	Red	0·40	–	Red-brown	0·54	–	Red-brown	0·38
ILA	–	Red	0·35	–	Red	0·27	–	Red	0·05
L-Histidinol	–	Red-orange	0·37	–	Red	0·58	–	Red	0·42
L-Histidine	–	Red	0·14	–	Red	0·21	–	Red	0·05
L-Tyrosine	–	Orange	0·45	–	Orange	0·36	–	Orange	0·10

Table 2. *Some characteristics of histidine-dependent mutants of Staphylococcus aureus*

Column I. Mutant is designated according to the scheme of Hartman, Loper & Serman (1960). *Column II.* The number of prototrophic colonies recovered on S agar from a 0·1 ml. sample of a 72 hr de-repression culture. TMTC = too many colonies to count. *Column III.* Growth responses on S agar containing several crystals of L-histidinol are recorded as negative (−), or stimulation (+). *Column IV.* Accumulations: 0 = no Pauly-positive imidazoles detected; 1 = 5-amino-1-ribosyl-4-aminoimidazole-carboxamide; 2 = imidazoleglycerol; 3 = imidazoleacetol; 5 = histidinol.

I Mutant	II Proto-trophic colonies (no.)	III Growth on L-histidinol	IV Accumu-lations
hisAb-1	0	+	1
hisB-2	1	+	1, 2
hisD-3	0	−	5
hisAb-5	2	+	1
hisAb-6	0	+	1
hisAb-7	0	+	1
hisD-8	0	−	1, 2, 5
hisGb-9	0	+	0
hisAb-10	0	+	1
hisAa-11	0	+	1
hisAb-12	0	+	1
hisGb-13	0	+	0
hisC-14	0	+	1, 2, 3
hisGb-15	1	+	0
hisD-16	TMTC	−	5
hisAb-17	1	+	1
hisD-18	0	−	5
hisAb-19	0	+	1
hisAb-20	0	+	1
hisC-23	0	+	1, 3
hisD-24	2	−	5
hisD-25	0	−	1, 2, 5
hisB-27	0	+	1, 2
hisG-29	TMTC	+	0
hisG-30	TMTC	+	0
hisAa-31	0	+	1
hisD-32	0	−	1, 5
hisD-33	0	−	1, 5
hisG-34	TMTC	+	0
hisG-35	TMTC	+	0
hisD-36	0	−	5
hisG-37	TMTC	+	0
hisGb-38	0	+	0
hisG-39	TMTC	+	0
hisGa-40	0	+	0
hisB-41	108	+	1, 2
hisD-42	0	−	5
hisD-43	0	−	5
hisD-44	0	−	5
hisG-45	6	+	0
hisD-46	0	−	5
hisD-47	0	−	5
hisC-48	0	+	1, 2, 3
hisE-49	0	+	0
hisC-50	0	+	1, 3
hisD-51	0	−	1, 2, 5
hisD-52	0	−	5
hisD-54	0	−	1, 5
hisD-55	0	−	5
hisC-56	0	+	1, 3
hisD-58	0	−	5
hisD-59	0	−	1, 5
hisD-60	0	−	5
hisD-61	0	−	5
hisGa-62	7	+	0
hisAaD-63	0	−	1
hisA-64	TMTC	+	1
hisG-65	TMTC	+	0
hisC-66	TMTC	+	1, 2, 3
hisD-68	5	−	5
hisD-69	0	−	5
hisD-70	0	−	5
hisD-71	0	−	5
hisD-72	0	−	1, 5
hisC-73	5	+	1, 3
hisGa-74	10	+	0
hisGa-75	0	+	0
hisAc-76	0	+	1
hisA-77	78	+	1
hisG-78	100	+	0
hisAa-79	0	+	1
hisA-80	0	+	1
hisD-81	0	−	5
hisD-82	TMTC	−	5
hisAa-83	0	+	1
hisD-84	0	−	5
hisD-85	0	−	5
hisG-87	263	+	0
hisD-88	TMTC	−	5
hisG-89	620	+	0
hisC-90	124	+	1, 3
hisC-91	0	+	1, 2, 3
hisE-93	2	+	0
hisG-94	410	+	0
hisG-95	TMTC	+	0
hisC-96	65	+	3
hisD-98	0	−	5
hisGb-99	3	+	0
hisD-100	0	−	5
hisC-101	120	+	3
hisD-103	TMTC	−	5
hisD-104	0	−	5

amounts of histidinol were detected when supernatant fluids were prepared from unshaken cultures.

A comparison of the imidazoles accumulated by representative mutants of *Staphylococcus aureus* and *Salmonella typhimurium* is shown in Fig. 1. One difference observed between mutants of these two species was the accumulation of AIC-R by *A* mutants of *S. aureus* and the accumulation of AIC by the corresponding *E*, *A*, *H* and *F* mutants of *S. typhimurium*. Small amounts of ILA were accumulated by *C* mutants of *S. typhimurium* but not by *C* mutants of *S. aureus*. Also, mutants of *S. typhimurium* invariably produced greater quantities of accumulations than did mutants of *S. aureus*.

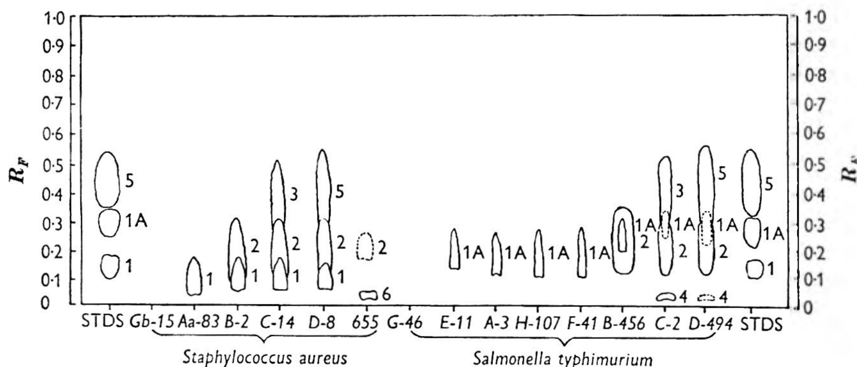
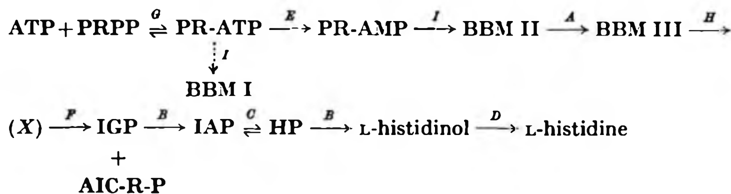


Fig. 1. Tracing of a chromatogram of Pauly-positive imidazoles accumulated by histidine-dependent mutants of *Staphylococcus aureus* and *Salmonella typhimurium*. The chromatogram was prepared on Whatman no. 3 MM filter paper and developed in *n*-butanol saturated with 2 N-NH_3 solution. Imidazoles were detected by spraying the chromatogram with diazo sulphanilic acid reagent followed by 5% (w/v) Na_2CO_3. Imidazoles were identified as follows 1 = 5-amino-1-ribosyl-4-imidazolecarboxamide; 1A = 5-amino-4-imidazolecarboxamide; 2 = imidazoleglycerol; 3 = imidazoleacetol; 4 = imidazolelactic acid; 5 = histidinol; 6 = histidine. The strains of *S. aureus* and *S. typhimurium* from which the imidazoles were obtained and the chemical standards (STDS) are indicated below the origin.

DISCUSSION

The pathway used by *Salmonella typhimurium* for the biosynthesis of L-histidine has been studied in detail (Ames, Garry & Herzenberg, 1960; Ames, Martin & Garry, 1961; Smith & Ames, 1964) and has been proposed to be as follows:



Based upon their failure to accumulate Pauly-positive imidazoles, the *G* and *E* mutants of *Staphylococcus aureus* are believed to correspond to the *G* and *I* mutants, respectively, of *Salmonella typhimurium*. Hartman *et al.* (1960) reported that *E*,

A, *H* and *F* mutants of *S. typhimurium* did not accumulate Pauly-positive imidazoles. In the present study, lyophilization of the supernatant fluids of cultures of these mutants permitted the detection of a compound which was indistinguishable from AIC. It is possible that AIC and AIC-R, the latter compound being accumulated by *A* mutants of *S. aureus*, could be derived as cleavage products of the BBM compounds. Both IG and IA, accumulated by *B* and *C* mutants of both species, are believed to be derived from their corresponding phosphate esters (Haas *et al.* 1952; Hartman, 1956). Histidinol, accumulated by *D* mutants of both species, is believed to be the only actual intermediate of L-histidine biosynthesis which has been identified in the culture fluids. This conclusion is supported by the observation that histidinol was the only imidazole among those accumulated by mutants of *S. typhimurium* which was utilized by mutants of *S. aureus*. The remaining imidazoles which were accumulated by mutants of *S. typhimurium* did not relieve the histidine requirement of mutants of *S. aureus* because they were either impermeable to the cell (i.e. phosphate esters: Hass *et al.* 1952; Hartman, 1956) or not actual intermediates in the pathway of histidine biosynthesis (i.e. IG and IA: Ames & Mitchell, 1955; Hartman, 1956).

The similarities observed in the Pauly-positive imidazoles accumulated by mutants of *Staphylococcus aureus* and *Salmonella typhimurium* and the similarities observed at the genetic level (Kloos & Pattee, 1965) support the conclusion that the pathways used for the biosynthesis of L-histidine are similar, if not identical, in both species. This conclusion is based upon the assumption that the imidazoles accumulated by *S. aureus*, like those accumulated by *S. typhimurium*, are modified intermediates of the pathway, and that, with the exception of AIC and AIC-R, the modifications are identical. Final conclusions about the pathway used in *S. aureus*, however, must await further studies to identify the specific substrates and products of the enzymes essential for the synthesis of L-histidine in this organism.

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Transduction Analysis of the Histidine Region in *Staphylococcus aureus*

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SUMMARY

The genetic control of histidine biosynthesis in *Staphylococcus aureus* was examined by an analysis of 66 histidine-dependent mutants by using stable and abortive transduction. These mutants, which previously had been differentiated into classes according to their biochemical characteristics, all occupied sites within a single linkage group, referred to as the histidine region. This region has been separated into six gene loci on the basis of complementation studies and the biochemical characteristics of the mutants. The order of genes within the histidine region, *E, A, B, C, D, G,* was determined from the results of reciprocal transductions to wild type and donor type. The order of mutant sites was also linearly related to the ability of these mutants to form histidine-independent transductants when infected with phage prepared on the parent strain. This observation, and the inequalities in reciprocal transduction frequencies obtained in inter-mutant transductions, support the hypothesis that all donor fragments which participate in transductions involving the histidine region are identical, and that the histidine region is located extremely close to one terminus of the donor fragment. Intergenic complementation occurred among mutants of all gene loci; in addition, three complementation units within the *A* gene and two complementation units within the *G* gene were detected.

INTRODUCTION

A genetic analysis of seven histidine-dependent mutants of *Neurospora crassa* revealed the existence of three linkage groups and four different gene loci (Haas, Mitchell, Ames & Mitchell, 1952). Webber & Case (1960) extended this analysis to include over 700 mutants of *N. crassa*. In these studies, representative mutants from each of seven biochemical groups were analysed for their linkage relationships and were found to represent seven different histidine loci which formed five linkage groups. In contrast to the wide distribution of histidine loci throughout the genome of *N. crassa*, a cluster of four adjacent histidine loci was found in *Salmonella typhimurium* (Hartman, 1956). Additional investigations were conducted which involved about 1000 histidine-dependent mutants (Hartman, Loper & Serman, 1960; Ames & Hartman, 1962, 1963). By means of transduction, a genetic map of the histidine operon was constructed which consisted of eight adjacent loci in the sequence *E, F, A, H, B, C, D, G.* By abortive transduction, Hartman, Hartman & Serman (1960) determined the functional relationships of over 200 histidine-dependent mutants. The results of this analysis divided the mutants into eight classes, *A* to *H*. In addition, intragenic complementation was found

to occur among the *E*, *B* and *D* mutants. The *E* and *B* genes were composed of four complementation units each (*a*, *b*, *c*, *d*). The *D* gene was composed of two complementation units (*a*, *b*). Loper (1960, 1961) analysed the *B* gene in more detail and found that 23 mutants of the *Bc* complementation unit lacked both the imidazoleglycerol phosphate dehydrase and the histidinol phosphate phosphatase enzymes, whereas a large number of *Ba*, *Bb* and *Bd* mutants lacked only the imidazoleglycerol phosphate dehydrase. More recently, Smith & Ames (1964) showed that *Ea* and *Ed* mutants could be differentiated from the *Eb* and *Ec* mutants. The former mutants now are designated *E* mutants, while the *Eb* and *Ec* mutants now are referred to as *I* mutants.

Ephrati-Elizur, Srinivasan & Zamenhof (1961) studied the histidine region of the chromosome of *Bacillus subtilis* by means of transformation. These investigators determined that twelve histidine-dependent mutants represented two loci, *A* and *D*. Because mutants of the *A* locus were not closely linked to the *D* locus, it was suggested that the region between these two loci might be occupied by genes which controlled other steps in the biosynthesis of L-histidine.

The present paper describes a transduction analysis of histidine-dependent mutants of *Staphylococcus aureus*. A unique method of mapping the histidine loci of *S. aureus* is described which is based upon the frequency with which the mutant strains are transduced to histidine independence when the parent strain is used as the donor.

METHODS

Media. The media used in this study included Brain Heart Infusion (BHI; Difco) agar, P and D broth (Pattee & Baldwin, 1961), S agar (Kloos & Pattee, 1965) and an enriched defined medium agar (ES agar) prepared by the addition of 1·0% (v/v) P and D broth to S agar. All glassware used in conjunction with the defined media was cleaned in chromic + sulphuric acid and thoroughly rinsed with deionized water before use.

Bacterial strains. This study was done exclusively with strain 655 of *Staphylococcus aureus* (Pattee & Baldwin, 1962) and with histidine-dependent mutants derived either from this strain or from a single penicillinase-deficient mutant of this strain. These mutants, the nomenclature used in their designation, and their biochemical characteristics are described by Kloos & Pattee (1965).

Transduction. In all instances bacteriologically-sterile lysates of phage 83 previously propagated on *Staphylococcus aureus* strain 655 (designated phage 83/655) or on appropriate mutants of strain 655 were used for transduction. The methods used to prepare these lysates were described by Pattee & Baldwin (1961). These lysates were prepared from phage 83 which had been maintained on the appropriate International strain of *Staphylococcus aureus*. Serial passage of phage 83 on *S. aureus* strain 655 was avoided as this practice resulted in a loss of transductional activity.

Cultures to be used as recipients in transduction experiments were grown on BHI agar slopes for 18 hr at 37°. Each culture was suspended in 1·0 ml. of P and D broth (coccal concentration = $5\text{–}7 \times 10^{10}$ cocci/ml.) and 0·5 ml. of this suspension transferred to a centrifuge tube containing 1·0 ml. P and D broth. To this suspension was added 0·5 ml. of the transducing phage lysate which had been diluted previously with P and D broth when necessary, to obtain a multiplicity of infection in the

transduction suspension of 2·0–3·5. Controls consisted of identical coccal suspensions, to which 0·5 ml. P and D broth was added in place of the phage lysate. The suspensions were shaken for 30 min. at 37° by means of a Burrell Wrist-Action Shaker (2223 Fifth Avenue, Pittsburg, Pa., U.S.A.) with the arms mounted over water baths. The maximum shaker setting of 10 was used. After shaking, the cocci were collected by centrifugation and resuspended in 1·0 ml. saline. Duplicate 0·1 ml. samples from a 1/10 dilution of each suspension were then spread over the surfaces of pre-dried ES agar plates. The plates were incubated at 37° for 48 hr, after which the average numbers of histidine-independent and donor-type transductants per sample were determined. Abortive transductants were detected by spreading 0·1 ml. samples of the undiluted transduction suspension over pre-dried plates of S agar. After incubation of these plates for 48 hr at 37°, they were examined with a dissecting microscope for abortive transductants, as indicated by the presence of 'minute' colonies (Ozeki, 1959).

In all experiments, the transduction frequency was recorded as the average number of colonies recovered from duplicate 0·1 ml. samples of the 1/10 dilution of the transduction suspension. The reversion frequency was recorded as the average number of colonies recovered from duplicate 0·1 ml. samples of the 1/10 dilution of the control suspension.

RESULTS

Effect of the multiplicity of infection on the transduction frequency

Among those bacteriophages of the International Typing Series which could be propagated to high titre on *Staphylococcus aureus* strain 655 (phages 83, 53, 54, 77, 47), phage 83 possessed the highest activity in transducing histidine independence to histidine-dependent mutants of strain 655. Preliminary studies with phage 83 showed that the multiplicity of infection used in the transduction suspension influenced the transduction frequency to a significant extent. With phage 83/655 and three recipient strains (*hisAb-1*, *hisD-8*, *hisGb-15*), when the multiplicity of infection was maintained between 2·0 and 3·5, maximum and reproducible transduction frequencies were obtained. Accordingly, all experiments were made with transduction suspensions infected at these multiplicities.

Recipient capacity, reversion frequency and incidence of abortive transduction

All histidine-dependent mutants were infected with phage 83/655 to determine the recipient capacity (efficiency to produce prototrophic transductants) and the reversion frequency of each mutant, and to determine which mutants were capable of undergoing abortive transduction. The experiment was made with a single lysate of phage 83/655 so that the titre of the transducing phage lysate could be eliminated as a factor responsible for variations in the recipient capacities of the mutants. The results of this experiment are shown in Table 1. The mutants listed in the legend of Table 1 were discarded from further use in genetic studies either because of their high reversion frequencies or because of their leakiness, or of both. Those mutants which were not analysed by transduction have been designated solely according to their biochemical characteristics (Kloos & Pattee, 1965). With the exception of mutant *hisAaD-63*, none of the *D* mutants gave rise to detectable abortive

transductants when coccal suspensions infected with phage 83/655 were inoculated on to either S agar or ES agar. However, after the S agar plates inoculated with phage-infected suspensions of *D* mutants had been incubated for 48 hr, the addition of a small amount of crystalline L-histidine (monohydrate; California Corporation

Table 1. *Frequency of transduction of histidine independence to histidine-dependent mutants of Staphylococcus aureus by phage 83/655*

Phage 83/655 was used to infect $2{\cdot}5$–$3{\cdot}5 \times 10^{10}$ cocci of each recipient at a multiplicity of 2·0–3·5. The transduction frequency is the average number of prototrophic transductants recovered per 0·1 ml. of a 1/10 dilution of the transduction suspension inoculated in duplicate on ES agar. The reversion frequency is the average number of prototrophic colonies recovered from uninfected coccal suspensions which were diluted and plated in the same manner as the transduction suspensions. The capacity of a mutant to undergo abortive transduction was determined on S agar. The presence of abortive transductants is indicated by a + symbol, and the failure of a mutant to give rise to abortive transductants without the addition of L-histidine is indicated by a − symbol. See text for details of the histidine stimulation procedure. Mutants found to be excessively leaky, or to revert at frequencies excessively high for use in genetic analyses, or both, include mutants *his: D-16, D-25, B-27, G-29, G-30, G-34, G-35, G-37, G-39, G-45, C-48, D-51, A-64, G-65, D-70, A-77, G-78, D-82, G-87, D-88, G-89, C-91, G-94, G-95, C-101* and *D-103*.

Mutant	Transduction frequency	Reversion frequency	Presence of abortive transductants	Mutant	Transduction frequency	Reversion frequency	Presence of abortive transductants
hisAb-1	136	2	+	*hisC-50*	368	2	+
hisB-2	332	14	+	*hisD-52*	509	2	−
hisD-3	474	0	−	*hisD-54*	529	5	−
hisAb-5	119	4	+	*hisD-55*	538	1	−
hisAb-6	125	2	+	*hisC-56*	370	12	+
hisAb-7	131	4	+	*hisD-58*	334	0	−
hisD-8	446	2	−	*hisD-59*	552	4	−
hisGb-9	1192	10	+	*hisD-60*	492	5	−
hisAb-10	136	10	+	*hisD-61*	343	0	−
hisAa-11	140	10	+	*hisGa-62*	1339	18	+
hisAb-12	151	1	+	*hisAaD-63*	67	0	+
hisGb-13	1368	2	+	*hisC-66*	482	55	+
hisC-14	401	3	+	*hisD-68*	590	0	−
hisGb-15	1300	2	+	*hisD-69*	616	0	−
hisAb-17	145	2	+	*hisD-71*	488	0	−
hisD-18	407	3	−	*hisD-71*	541	3	−
hisAb-19	113	1	+	*hisC-73*	322	7	+
hisAb-20	142	2	+	*hisGa-74*	924	10	+
hisC-23	518	15	+	*hisGa-75*	1170	3	+
hisD-24	419	1	−	*hisAc-76*	141	0	+
hisAa-31	132	3	+	*hisAa-79*	152	3	+
hisD-32	503	0	−	*hisA-80*	182	60	+
hisD-33	499	1	−	*hisD-81*	359	0	−
hisD-36	397	1	−	*hisAa-83*	127	2	+
hisGb-38	1389	17	+	*hisD-84*	506	0	−
hisGa-40	1336	23	+	*hisD-85*	627	8	−
hisB-41	215	2	+	*hisC-90*	328	1	+
hisD-42	677	4	−	*hisE-93*	100	27	+
hisD-43	457	1	−	*hisC-96*	358	20	+
hisD-44	425	0	−	*hisD-98*	347	2	−
hisD-46	513	28	−	*hisGb-99*	990	23	+
hisD-47	575	2	−	*hisD-100*	361	0	−
hisE-49	118	7	+	*hisD-104*	434	0	−

Table 2. *Transduction analysis of the linkage relationships of the A, B, C, D and G mutants of Staphylococcus aureus by the 'best-fit' method*

Phage 83 prepared on each donor was used to infect 2·5–3·5 × 10^{10} cocci of each recipient at a multiplicity of 2·0–3·5. The transduction frequency is the average number of prototrophic transductants recovered per 0·1 ml. of a 1/10 dilution of the transduction suspension inoculated in duplicate on ES agar. The reversion frequency is the average number of prototrophic colonies recovered from uninfected coccal suspensions which were diluted and plated in the same manner as the transduction suspensions.

Recipient	Reversion frequency	Transduction frequencies obtained using donor													
		655	*hisAa-83*	*hisAb-5*	*hisAb-10*	*hisB-2*	*hisC-90*	*hisC-14*	*hisC-23*	*hisD-36*	*hisD-8*	*hisD-42*	*hisGa-75*	*hisGa-74*	*hisGb-15*	*hisAaD-63*
hisAa-83	2	127	2	9	16	27	47	48	60	57	64	95	76	99	91	3
hisAb-5	4	119	30	1	5	27	32	35	42	39	45	99	79	95	76	15
hisAb-10	10	136	32	11	9	29	35	39	51	45	52	93	83	93	94	20
hisB-2	14	332	189	153	124	19	76	100	155	160	155	211	265	238	257	103
hisC-90	1	328	195	139	136	107	1	10	26	34	33	112	134	134	114	34
hisC-14	3	401	276	298	197	184	19	4	13	25	41	66	131	104	113	28
hisC-23	15	518	423	392	362	328	93	69	8	50	90	203	228	230	250	82
hisD-36	1	397	236	307	212	160	123	49	17	3	15	33	59	81	62	29
hisD-8	2	446	257	342	248	208	152	92	52	25	2	31	67	52	57	0
hisD-42	4	667	491	504	365	364	218	161	102	37	24	2	139	143	161	37
hisGa-75	3	1170	978	1102	851	769	564	648	621	554	504	524	0	28	122	503
hisGa-74	10	924	856	1014	812	781	550	667	641	620	455	482	24	6	81	586
hisGb-15	2	1300	892	1059	834	955	600	764	627	573	445	557	75	48	2	532
hisAaD-63	0	67	1	2	4	1	2	2	1	1	0	1	11	12	13	0

for Biochemical Research, Los Angeles, Calif.) to the agar surface permitted detectable growth of the abortive transductants.

Determination of the order of mutant sites within the histidine region by the 'best-fit' method

The order of mutant sites within the histidine region was determined by the 'best-fit' method (Hartman, Loper & Serman, 1960). In this method, the frequencies with which all possible pairs of mutants underwent recombination, as determined by transduction, were used to define a single order of mutant sites which best agreed with all the data. In the present study, three mutants from each class were used in the 'best-fit' method of analysis, with the exceptions of class *E* which was omitted, and class *B* for which only one mutant was suitable for use. These mutants were chosen for their low reversion frequencies and absence of leakiness, although mutants *hisAa-83* and *hisC-90* were slightly leaky; mutant *hisAaD-63* also was included in the experiment. Lysates of phage 83 prepared on each mutant and on *Staphylococcus aureus* strain 655 were used to infect each mutant, and the numbers of transductants obtained on ES agar were determined. The results of this experiment are shown in Table 2. The order of mutants as they are shown in Table 2, with the exception of mutant *hisAaD-63*, represents the order of mutant sites as determined from the results of this experiment. The histidine-dependent mutants exhibited considerable variation in recipient capacity, as is evident from the transduction frequencies obtained with phage 83/655. For this reason, an analysis of the transduction data after correcting it for variations in recipient capacity was not made. Instead, all the transduction frequencies obtained with a single recipient were analysed for the most probable order of mutant sites, and this analysis repeated for each recipient. These results were then compiled to determine the order of mutant sites which best satisfied all the data. The results indicate that those mutants which exhibited similar accumulation patterns (Kloos & Pattee, 1965) were also closely linked to one another. From a consideration of the results obtained with mutant *hisAaD-63*, this mutant appears to be a double mutant. This conclusion is supported by the observation that this mutant accumulated 5-amino-1-ribosyl-4-imidazolecarboxamide, but did not utilize exogenous L-histidinol (Kloos & Pattee, 1965) and by the results of complementation studies which are presented below.

Determination of the position of mutant sites relative to the D locus by the ratio test

To obtain further information concerning the arrangement of mutant sites within the histidine region, all single-site mutants used previously in the 'best-fit' analysis, as well as mutant *hisE-49*, were analysed by the ratio test (Hartman, Loper & Serman, 1960). In the ratio test, the numbers of donor-type and wild-type transductants obtained after infection of one mutant with phage grown on another mutant were determined simultaneously from samples of the transduction suspension spread on ES agar. The results are expressed in terms of the probability (p) of integration, by the recipient, of the wild-type allele of the donor without the simultaneous integration of the mutant allele of the donor. Therefore, p is equal to the numbers of wild-type transductants divided by the sum of the wild-

type and donor-type transductants. Mutants *hisD-36*, *hisD-8* and *hisD-42* were chosen for use as recipients since they secrete sufficient histidinol into the environment to support the growth of all other mutant classes. After infection of the *D* mutants with lysates of phage 83 prepared on other mutants, samples of the transduction suspension were spread on ES agar in the usual manner. Wild-type transductants were differentiated from donor-type transductants by their larger size and more intense pigmentation. The results of this experiment (Table 3) agree with the order of mutant sites as determined by the 'best-fit' method of analysis. Moreover, the results provide conclusive evidence for the existence of the entire histidine region as a single linkage group which is carried on a single donor fragment during transduction. Evidence that the smaller weakly-pigmented colonies were donor-type transductants includes the following facts: (1) no donor-type transductants were obtained when any *D* mutant was infected with phage prepared on any other *D* mutant; (2) the donor-type transductants were stimulated by L-histidinol (dihydrochloride; Mann Research Laboratories, New York, N.Y.); (3) the donor-type transductants did not undergo recombination with the strain from which their mutant allele was acquired, and yet did produce wild-type recombinants at predictable frequencies when they were transduced with phage 83 prepared on other mutants.

Table 3. *The probability (p) of independent integration of different histidine markers into class D mutant recipient strains of Staphylococcus aureus*

Phage 83 prepared on each donor was used to infect $2 \cdot 5$–$3 \cdot 5 \times 10^{10}$ cocci of each recipient at a multiplicity of 2·0–3·5. The numbers of wild-type and donor-type transductants recovered from 0·1 ml. samples of 1/10 dilutions of the transduction suspension spread on ES agar were determined from duplicate platings. The numbers (no.) columns indicate the total number of transductants scored to determine the value of p in each experiment. (p = numbers of wild-type transductants divided by the numbers of wild-type + donor-type transductants.)

	Recipients used					
	hisD-36		*hisD-8*		*hisD-42*	
Donor	p	No.	p	No.	p	No.
hisE-49	0·86	289	0·85	266	0·93	532
hisAa-83	0·84	267	0·85	300	0·88	554
hisAb-5	0·77	399	0·80	426	0·85	589
hisAb-10	0·79	267	0·77	322	0·83	421
hisB-2	0·56	290	0·60	347	0·63	737
hisC-90	0·50	246	0·45	333	0·53	377
hisC-14	0·12	406	0·22	427	0·27	594
hisC-23	0·05	350	0·11	452	0·16	651
hisGa-75	0·17	268	0·19	332	0·21	673
hisGa-74	0·21	462	0·20	508	0·19	711
hisGb-15	0·27	259	0·20	256	0·23	695

Complementation studies

All the histidine-dependent mutants produced abortive transductants when infected with phage 83/655, although the *D* mutants required the delayed addition of L-histidine to the S agar plates to facilitate their development. That the minute colonies were the result of abortive transduction is supported by their morphology

(0·02–0·10 mm. diam.), their failure to appear when a mutant was infected with phage 83 previously prepared on that mutant, and their failure to produce more than a single minute colony when subcultured on S agar plates.

To characterize the functional units of the histidine region, the mutants used in the 'best-fit' analysis were examined for their ability to complement one another. A number of additional mutants were included in this experiment when preliminary studies suggested that intragenic complementation occurred among the *A* and *G* mutants, and also that the *E* and *G* mutants, which did not accumulate Pauly-positive imidazoles (Kloos & Pattee, 1965) could be differentiated by their position within the histidine region. The results of this experiment did not show intragenic complementation within either the *C* gene (mutants *hisC: -90, -14, -23* were tested) or the *D* gene (mutants *hisD: -36, -8, -42, hisAaD-63* were tested); but the significance of this result is limited by the numbers of mutants of each class examined. The *A* gene was found to consist of three complementation units (*hisAa: -11, -31, -79, -83, hisAa D-63; hisAb: -1, -5, -6, -7, -10, -12, -17, -19, -20; hisAc-76*) while the *G* gene consisted of two complementation units (*hisGa: -40, -62, -74, -75; hisGb: -9, -13, -15, -38, -99*). In addition, all genes (*E, A, B, C, D* and *G*) were capable of intergenic complementation with all other genes controlling the biosynthesis of histidine.

Table 4. *Transduction analysis of the linkage relationships of the E, A and B mutants of Staphylococcus aureus by the 'best-fit' method*

Phage 83 prepared on each donor was used to infect $2{\cdot}5$–$3{\cdot}5 \times 10^{10}$ cocci of each recipient at a multiplicity of 2·0–3·5. The transduction frequency is the average number of prototrophic transductants recovered per 0·1 ml. of a 1/10 dilution of the transduction suspension inoculated in duplicate on ES agar. The reversion frequency is the average number of prototrophic colonies recovered from uninfected coccal suspensions which were diluted and plated in the same manner as the transduction suspensions.

		Transduction frequencies obtained using donor:							
Recipient	Reversion frequency	655	*hisE-49*	*hisAa-31*	*hisAa-11*	*hisAb-5*	*hisAb-10*	*hisAc-76*	*hisB-2*
hisE-49	7	118	0	10	20	35	21	28	36
hisAa-31	3	132	42	0	20	39	34	32	68
hisAa-11	10	140	44	17	9	41	30	38	60
hisAb-5	4	119	72	64	60	3	2	10	26
hisAb-10	10	136	65	51	52	11	6	16	23
hisAc-76	0	141	66	62	60	26	6	0	10
hisB-2	14	332	331	288	295	242	152	36	16

The order of E, A, and B genes and the order of genes controlling histidine biosynthesis

To determine the order of complementation units within the *A* gene and to verify the location of the *E* gene, a second transduction analysis was made by the 'best-fit' method. The results of this experiment (Table 4) established the order of genes and complementation units to be *E, Aa, Ab, Ac, B*. The precise order of mutant sites within the complementation units could not be determined unambiguously from the data. As an example, mutants *hisAb-5* and *hisAb-10* could exist in an order other than that used to list the mutants in Table 4.

Based upon the results of the 'best-fit' analyses, the ratio tests, and the complementation analysis, the histidine region of the chromosome of *Staphylococcus aureus* consists of the genetic arrangement depicted in Fig. 1. Included in Fig. 1 is the tentative pathway used by *S. aureus* for the biosynthesis of L-histidine as determined by the accumulation studies (Kloos & Pattee, 1965).

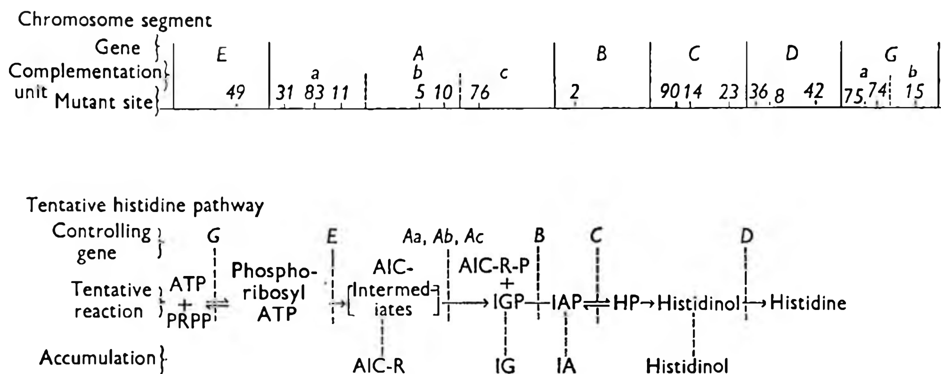

Fig. 1. A genetic map of the histidine region of the chromosome of *Staphylococcus aureus*, and the pathway used for the biosynthesis of histidine in this bacterium. Abbreviations used are: ATP = adenosine triphosphate; PRPP = phosphoribosylpyrophosphate; AIC = 5-amino-4-imidazolecarboxamide; AIC-R = 5-amino-1-ribosyl-4-imidazolecarboxamide; AIC-R-P = 5-amino-1-(5′-phosphoribosyl)-4-imidazolecarboxamide; IGP = imidazoleglycerol phosphate; IG = imidazoleglycerol; IAP = imidazoleacetol phosphate; IA = imidazoleacetol; HP = histidinol phosphate.

DISCUSSION

In the previous paper (Kloos & Pattee, 1965) evidence was presented that the biosynthesis of L-histidine in *Staphylococcus aureus* proceeds via a pathway which closely resembles the pathway found in *Salmonella typhimurium*. The results of the present study have shown that a corresponding similarity between the two species exists with respect to the genetic control of histidine biosynthesis. It is apparent that certain features of the histidine region of the chromosome of *S. aureus* require further study, particularly the *B* and *E* genes, each of which has been represented in the present work by only two mutants. None the less, sufficient information is available to permit certain comparisons to be made concerning the two species. The *A* gene of *S. aureus*, defined by a study of accumulations (Kloos & Pattee, 1965) consists of three complementation units as demonstrated by abortive transduction. It is most probable that this apparent intragenic complementation within the *A* gene is a reflexion of the fact that these complementation units actually are three different gene loci which correspond in function to one or more of the *E*, *A*, *H* or *F* genes of *S. typhimurium*. This possibility is supported by the close resemblance of the *A* mutants of *S. aureus* to the *E*, *A*, *H* and *F* mutants of *S. typhimurium* with respect to their accumulations. Clarification of this question can be obtained by a study of the enzymes which are affected by mutations within the various complementation units of the *A* gene of *S. aureus*. The demonstration of complementation within the *G* gene of *S. aureus*, and the absence of a similar phenomenon in *S. typhimurium* (Ames & Hartman, 1963), represent a definite difference between the two species. This observation is of some interest in view of the demonstration by

Martin (1963) that the enzyme produced under the control of the *G* gene in *S. typhimurium* possesses a subunit structure.

One of the most striking features of the histidine region in *Staphylococcus aureus* is the correlation which exists between the position of a mutant site within the histidine region and the frequency with which that mutant is transduced to histidine independence by the parent strain (i.e. recipient capacity). This relationship is indicated by the data shown in Table 1, which has been summarized according to mutant class in Table 5. When the mutants are arranged in an order of increasing recipient capacity, the order of mutant sites is in close agreement with the order determined by the ratio test and the 'best-fit' method of analysis. While there is some overlapping between the limits of recipient capacity of mutants of adjacent classes, the average recipient capacity for the mutant classes increases in a linear order which is identical with the order of gene loci. The only major discrepancy in this order is the overlapping which occurs between mutants of classes *C* and *D*. This overlapping is explained by the sensitivity of *S. aureus* strain 655 and mutants of this strain to L-histidinol (Kloos & Pattee, 1965), and the secretion of histidinol into the culture medium by the *D* mutants, which may have caused a decrease in the observed transduction frequencies below the actual values.

Table 5. *The relationship between the complementation unit or gene and the recipient capacity of histidine-dependent mutants of Staphylococcus aureus*

Recipient capacity is expressed in terms of the transduction frequencies obtained with phage 83/655. Phage 83/655 was used to infect $2{\cdot}5$–$3{\cdot}5 \times 10^{10}$ cocci of each recipient at a multiplicity of 2·0 to 3·5. The transduction frequency is the average number of prototrophic transductants recovered per 0·1 ml. of a 1/10 dilution of the transduction suspension inoculated in duplicate on to ES agar.

Complementation unit or gene	No. of mutants tested	Recipient capacity	
		Range	Mean
E	2	100–118	109
Aa	4	127–152	138
Ab	9	113–151	133
Ac	1	141	141
B	2	215–332	273
C	8	322–518	393
D	29	334–677	481
Ga	4	924–1339	1192
Gb	5	990–1389	1248

As a result of transduction studies in *Salmonella typhimurium* Ozeki (1959) suggested that the donor fragments which participate in transduction by phage PLT 22 are uniform and predetermined in size. While it is realized that other explanations exist, the theory of Ozeki (1959) appears best to explain the direct linear relationship which exists between the position of a mutant site within the histidine region and the recipient capacity of that mutant, and the inequalities obtained in reciprocal transduction between pairs of mutants. According to this hypothesis, and based on the data of the present study, the chromosome of *Staphylococcus aureus* always fragments immediately to the 'left' of the *E* gene of the histidine region (Fig. 1) during formation of the donor fragments. The result would be that all, or nearly all, of the donor fragments which participate in transductions involving

the histidine region would include the entire histidine region near this end of the fragment and in the same orientation. The donor fragment is assumed to extend beyond the 'right' end of the histidine region for a considerable distance, as evidenced by the high recipient capacity values obtained with the *G* mutants. Therefore, the frequency of transduction of a mutant by the parent strain is determined by the position of that mutant with respect to the terminus of the donor fragment (Fig. 2). A mutant whose wild-type allele is located near the terminus of the donor fragment, such as mutant *hisAa-83* (Fig. 2*A*), permits crossing over to

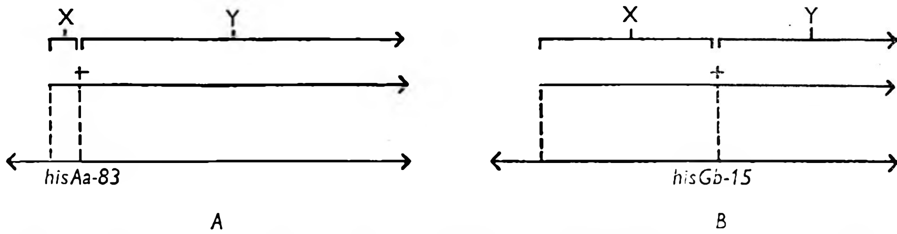

Fig. 2. Diagrammatic representation of the positions of the mutant sites in transductions of *Staphylococcus aureus* mutants *hisAa-83* and *hisGb-15* by the parent strain. The lower line represents the recipient chromosome and the upper line the donor fragment. The distances between the mutant sites and the terminal points of the donor fragment are designated by the letters X and Y.

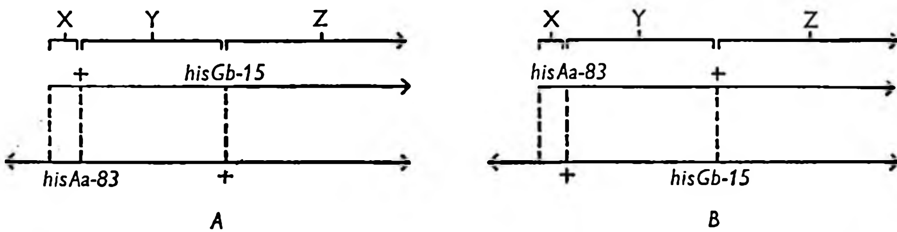

Fig. 3. Diagrammatic representation of the positions of the mutant sites in reciprocal transductions between *Staphylococcus aureus* mutants *hisAa*-83 and *hisGb*-15. The lower line represents the recipient chromosome and the upper line the donor fragment. The distances between the two mutant sites and the distances between the mutant sites and the terminal points of the donor fragment are designated by the letters X, Y and Z.

occur to the 'left' of the mutant site only within the small distance X. Mutants such as *hisGb-15* (Fig. 2*B*), whose wild-type allele is located at a greater distance from the terminus of the donor fragment, permit greater opportunity for cross-over to occur within distance X. Distance Y in both instances is sufficiently great that no detectable restriction in cross-over occurs to the 'right' of the recipient mutant site. Fig. 3 illustrates the effect of this situation on the results of inter-mutant transductions, in which inequalities in the frequencies of recombination in reciprocal crosses are observed. When mutant *hisAa-83* is used as a recipient with mutant *hisGb-15* (Fig. 3*A*), one cross-over within distance X and another within distance Y are required. When mutant *hisAa-83* is used as the donor in the same genetic cross (Fig. 3*B*), one cross-over must occur within distance Y, and the other within distance Z. Thus, in crosses involving two mutants, the frequency with which prototrophic recombinants are obtained is restricted by distance Y in all cases,

and by distance X separating the recipient mutant site from the terminus of the donor fragment when the recipient mutant site is closer to the terminus of the donor fragment than is the donor mutant site. This effect is reflected in the inequalities of reciprocal transduction frequencies, especially when these frequencies result from crosses between *A* and *G* mutants, where distance Y is maximal and distance X is minimal.

According to the above hypothesis, the incidence of abortive transduction obtained with all mutants after infection with phage propagated on the parent strain should be constant. While extensive data concerning this point has not been obtained, preliminary results confirm this prediction. In addition, the restriction in recombination due to the position of the histidine region within the donor fragment may affect the results of the ratio test. When the results of the ratio test are compared with the data of Hartman, Loper & Serman (1960), the ratios obtained in the present study are significantly higher than those obtained in similar experiments in *Salmonella typhimurium.* As would be expected, this difference is most apparent in crosses in which *A* and *E* mutants are used as donors and *D* mutants are used as recipients.

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The Transfer of Two Episomes, Colicinogenic Factor I and Resistance Transfer Factor, in *Shigella flexneri* Strains by Crosses Between Strains each Possessing a Single Episome

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SUMMARY

It was shown that bilateral transfer of two episomes *col I* and RTF, carried singly in different strains of *Shigella flexneri*, could occur at the same time. The transfer of *col I* occurred at a considerably higher frequency than did the transfer of RTF. Whilst the presence of *col I* in the acceptor organism appeared not to affect the frequency of transfer of RTF it was observed that, in some cases at least, the presence of RTF in the acceptor organism lowered the frequency of *col I* transfer.

INTRODUCTION

The term episome was proposed by Jacob & Wollman (1958) to designate certain genetic elements that may be present in or absent from a cell and, when present, may be either autonomous in the cytoplasm or integrated with the chromosome. According to this definition, colicinogenic factors can be designated as episomes. The subject of colicinogenic factors and colicines was reviewed by Frédéricq (1957, 1963). The colicinogenic factor has been shown to be transferred to non-colicinogenic cells by cell contact (Frédéricq, 1954). Resistance transfer factors (RTF), reviewed by Watanabe (1963), were considered by Watanabe & Fukasawa (1960) to be episomes and it was shown by these authors (Watanabe & Fukasawa, 1961) that these factors could be transmitted to different genera of Enterobacteriaceae by conjugation. It was observed by Watanabe & Fukasawa (1962) that the frequency of transfer of colicinogenic factor E1 (*col E1*) was very much decreased by the presence of RTF in donor strains. Frédéricq (1954) had shown that this colicinogenic factor could be transmitted only by F^+ (male) cells of *Escherichia coli*. On the other hand, *col I* does not require *F* factor for its transfer (Ozeki & Howarth, 1961; Clowes, 1961) and Watanabe & Fukasawa (1962) found that RFT in colicinogenic donor organisms did not suppress the transfer of *col I* in *E. coli* K-12. It had been shown that RTF, like *col I*, did not require the presence of *F* factor for its transmission (Mitsuhashi *et al* 1960). The fact that both *col I* and RFT can promote their own transfer enables an experimental system to be constructed in which crosses can be carried out completely independently of the *F* fertility factor episome. (Both *col I* and RTF are also fertility factors, as shown by their ability to promote their own transfer.) Watanabe & Fukasawa (1962) studied the interactions between RTF and *col I* in *E. coli* and found that the transfer of *col I* was

not affected by the presence of RTF, when both episomes were present in the same cell. We wished to investigate the interactions of these same two episomes in various strains of *Shigella flexneri* when the episomes were carried singly in organisms of different serotype and to measure the frequency of transfer of both episomes at the same time.

METHODS

Organisms. The strains of *Shigella flexneri* and their biochemical reactions are shown in Table 1. *Escherichia coli* K-12, CSH-2-222, received from Dr T. Watanabe, was used as a source of RTF, conferring resistance to streptomycin, chloramphenicol, tetracycline and sulphonamide. This *E. coli* strain was female (F^-) and methionineless (met^-). (The RTF had originally been derived from a strain of *S. flexneri* type 2b.) This RTF was introduced to *S. flexneri* 1a1 and 1a2 by overnight growth in mixed broth culture at 37°. *S. flexneri* strains 2b, 4a and 5a carrying *col I* were derived from *S. flexneri* 1a1 which was colicinogenic (Mulczyk, unpublished).

Table 1. *Characteristics of Shigella flexneri strains*

Strain designation	Source	Serotype	Acid in 48 hr at 37°		Indole in 48 hr at 37°	Catalase
			Maltose	Arabinose		
1a1	NCTC (8192)	1a	+	+	−	+
1a2	NCTC (3)	1a	+	+	+	+
2b	NCTC (8518)	2b	−	+	−	+
4a	Edinburgh, 1955	4a	−	−	+	+
5a	NCTC (8523)	5a	+	±	+	+

Media. The liquid medium was Difco heart infusion broth. Strains containing RTF were selected from mixed cultures by growth on Difco heart infusion agar containing streptomycin 250 μg./ml. (All strains growing on this concentration of streptomycin also grew in the presence of chloramphenicol 100 μg./ml., tetracycline 100 μg./ml. and sulphathiazole 500 μg./ml).

Crosses. Crosses were performed in broth. Donor and acceptor strains, grown for 48 hr at 37°, were mixed in a ratio of 1:1 (3·5 + 3·5 ml.) and incubated for 48 hr at 37°. After incubation the mixed culture was diluted and 0·1 ml. portions of suitable dilutions were spread uniformly on agar (for total viable count) and on agar containing streptomycin. The plates were incubated for 48 hr at 37° and, after scoring, were replicated to streptomycin agar with velveteen pads (Lederberg & Lederberg, 1952) in order to retain colonies for serological and biochemical tests. After replication the plates were exposed to chloroform and overlayed with agar. When dry the surface of the plates was flooded with a culture of an indicator organism, *Escherichia coli* strain Row, which was sensitive to colicin I. The number of colonies that were colicinogenic was determined after overnight incubation at 37°.

Serology. Serological agglutination tests were done on slides with type-specific sera obtained from Standards Laboratory for Serological Reagents, Central Public Health Laboratory, London.

Calculation of frequency of transfer of episomes

(*a*) *For cross* col^{-} *RTF*$^{+} \times col^{+}$ *RTF*$^{-}$

Let total viable count on plain agar $= a$.

Let total count on streptomycin agar $= b$, (i.e. total number of cells containing RTF^{+}.)

Let number of RTF^{+} cells which were also $col^{+} = c$.

Then number of RTF^{+} cells which were $col^{-} = b-c$.

Let percentage of RTF^{+} col^{+} cells which have the serotype of the first parent be $d\,\%$.

Then number of RTF^{+} col^{+} cells which have newly acquired $col^{+} = cd/100$.

So number of RTF^{+} col^{+} cells which have newly acquired RTF^{+} $= [c(100-d)]/100$.

The percentage frequency of col^{+} transfer (estimated from the total input of RTF^{+} col^{-} cells) is

$$\frac{cd/100}{b-[c-(cd/100)]} \times 100\,\%$$

$$= \frac{cd}{b-[c-(cd/100)]}\,\%.$$

The percentage frequency of RTF^{+} transfer (estimated from the total input of RTF^{-} col^{+} cells) is

$$\frac{[c(100-d)]/100}{a-b-[c-(cd/100)]} \times 100\,\%$$

$$= \frac{c(100-d)}{a-b-[c-(cd/100)]}\,\%.$$

(*b*) *For cross* col^{-} *RTF*$^{+} \times col^{-}$ *RTF*$^{-}$

Let total viable count on plain agar $= x$.

Let total count on streptomycin agar $= y$.

Let number of cells which have newly acquired RTF^{+} $= z$.

Then number of RTF^{+} parental cells $= y-z$.

Number of RTF^{-} parental cells $= x-(y-z)-z = x-y$.

The percentage frequency of RTF^{+} transfer $= z/(x-y) \times 100\,\%$.

RESULTS

The results obtained in crosses between two strains of *Shigella flexneri*, one of which carried *col I* and the other RTF, are presented in Table 2. It can be seen that a strain which acted as a donor of RTF also acted as an acceptor of *col I* and vice versa. In all of the crosses there was transfer of *col I* to acceptor strains with a fairly high frequency. However, in the same crosses the frequency of transfer of RTF was either very low or undetectable in the numbers tested. These results show the frequency of transfer of both *col I* and RTF to acceptors possessing only the other factor.

We also investigated crosses in which RTF was transferred to acceptor strains that were non-colicinogenic and streptomycin sensitive (as well as being sensitive to chloramphenicol, tetracycline and sulphathiazole). The results of these experi-

ments are shown in Table 3. The frequency of transfer of RTF was again very low but not lower than when *col I* was present in the acceptor organisms.

Twenty recombinant colonies from each of the crosses described in Tables 2 and 3 were tested for serotype and biochemical properties. All had the biochemical properties typical of their serotype (given in Table 1) showing that no transfer of antigen had taken place.

Table 2. *RTF and col I transfer between strains of Shigella flexneri involving RTF and col I carried singly*

Cross	% organisms of serotype 1 that acquired *col*$^{+}$	No. tested*	Range of results (%)	No of experiments
1a1 *col*$^{-}$ RTF^{+} × 2b *col*$^{+}$ RTF^{-}	52	200	33–74	3
1a2 *col*$^{-}$ RTF^{+} × 2b *col*$^{+}$ RTF^{-}	25	200	19–34	4
1a1 *col*$^{-}$ RTF^{+} × 4a *col*$^{+}$ RTF^{-}	6	200	1–12	3
1a2 *col*$^{-}$ RTF^{+} × 4a *col*$^{+}$ RTF^{-}	25	200	22–30	4
1a1 *col*$^{-}$ RTF^{+} × 5a *col*$^{+}$ RTF^{-}	13	600	7–20	4
1a2 *col*$^{-}$ RTF^{+} × 5a *col*$^{+}$ RTF^{-}	34	600	6–70	10
1a1 *col*$^{-}$ RTF^{-} × 5a *col*$^{+}$ RTF^{-}	98	1000	86–99	10

Cross	% organisms of serotype 2 or 4 or 5 that acquired RTF^{+}	No. tested†	Range of results (%)	No. of experiments
1a1 *col*$^{-}$ RFT^{+} × 2b *col*$^{+}$ RTF^{-}	0·04	200	0·03–0·06	3
1a2 *col*$^{-}$ RTF^{+} × 2b *col*$^{+}$ RTF^{-}	0·001	200	0–0·003	4
1a1 *col*$^{-}$ RTF^{+} × 4a *col*$^{+}$ RTF^{-}	0·0	200	—	3
1a2 *col*$^{-}$ RTF^{+} × 4a *col*$^{+}$ RTF^{-}	0·0	200	—	4
1a1 *col*$^{-}$ RTF^{+} × 5a *col*$^{+}$ RTF^{-}	0·01	600	0–0·02	4
1a2 *col*$^{-}$ RTF^{+} × 5a *col*$^{+}$ RTF^{-}	0·01	600	0–0·1	10
1a1 *col*$^{-}$ RTF^{-} × 5a *col*$^{+}$ RTF^{-}	—	—	—	—

* = number of *col*$^{+}$ RTF^{+} colonies tested for serotype.
† = number of RTF^{+} colonies tested for serotype.

Table 3. *Transmission of RTF to non-colicinogenic Shigella flexneri cells*

Cross	% organisms of serotype 2 or 4 or 5 that acquired RTF^{+}	No. tested*	Range of results (%)	No. of experiments
1a1 *col*$^{-}$ RTF^{+} × 2b *col*$^{-}$ RTF^{-}	0·05	200	0·03–0·1	4
1a2 *col*$^{-}$ RTF^{+} × 2b *col*$^{-}$ RTF^{-}	0·0	200	—	4
1a1 *col*$^{-}$ RTF^{+} × 4a *col*$^{-}$ RTF^{-}	0·009	200	0·0–0·03	4
1a2 *col*$^{-}$ RTF^{+} × 4a *col*$^{-}$ RTF^{-}	0·0	200	—	4
1a1 *col*$^{-}$ RTF^{+} × 5a *col*$^{-}$ RTF^{-}	0·06	600	0·0–0·14	10
1a2 *col*$^{-}$ RTF^{+} × 5a *col*$^{-}$ RTF^{-}	0·09	600	0·0–0·16	10

* = number of RTF^{+} colonies tested for serotype.

DISCUSSION

On the basis of our results it would appear that the presence of *col I* in acceptor strains of *Shigella flexneri* had no effect on RTF transfer. In all crosses, whether the RTF acceptor strains were colicinogenic or not, the frequency of RTF transfer

was always very low and in some cases was not detectable. A comparison of the frequency of transfer of *col I* to RTF-containing acceptor strains with results obtained using a non-RTF-containing acceptor strain (Table 2) indicated that, at least in some cases, the presence of RTF in an acceptor organism decreased the frequency of *col I* transfer. The observed high frequency of transfer of *col I* into wild-type acceptor cells has been attributed (Mulczyk, unpublished) to epidemic spread of the colicinogenic factor in the acceptor population. The reason for the lower frequency of transfer when the acceptor cells contain RTF is not known; it is possible that the presence of RTF prevents epidemic spread from occurring.

In all crosses investigated, the low frequency of transfer of RTF might indicate that epidemic spread of RTF does not occur in *Shigella flexneri*. Watanabe (1963) quoted unpublished data which suggested that, in *Escherichia coli*, the low transferability of RTF could be accounted for by specific characters of the strains being used; of these characters, little or nothing was known. It is possible that the low frequency of transfer we observed in *S. flexneri* may also be accounted for by unknown characters present in the strains we used.

Amongst the recombinant strains isolated from some of the crosses involving both *col I* and RTF were some colonies in which the phenotypic expression of colicinogeny was suppressed when first tested on streptomycin agar. That it was genotypically present was shown by the fact that after one transfer in broth, followed by plating to streptomycin agar, it was possible to isolate, in some cases, colicinogenic colonies. This phenomenon will be examined in greater detail later.

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'Substrate-Accelerated Death' of *Aerobacter aerogenes*

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SUMMARY

'Substrate-accelerated death' (Postgate & Hunter, 1963*a*, 1964) was observed with carbon-limited but not ammonium-, phosphate- or sulphate-limited *Aerobacter aerogenes* grown at 37° in defined medium and starved at 37° in aerated saline buffers containing the growth-limiting substrate. Carbon sources besides the one limiting growth increased the death-rate of starved mannitol-, glycerol-, galactose- and ribose-limited bacteria. Glycerol-accelerated death depended on the rate of oxidation of glycerol and the bacterial concentration; with bacteria fully adapted to glycerol, populations of less than $1–2 \times 10^9$ organisms/ml. died at a faster rate the denser the population and above this concentration the death-rate decreased with increasing bacterial concentration. Death was delayed when aerated bacterial suspensions containing glycerol were dialysed at 37° against saline buffer containing the substrate. Bacteria-free filtrates, from populations dying in the presence of glycerol, accelerated the death of fresh bacteria to a greater extent than did glycerol alone. In contrast, bacteria-free filtrates from dense populations surviving in the presence of glycerol partially protected fresh bacteria exposed to glycerol. Mg^{2+} abolished glycerol-accelerated death but not the lethal effect of filtrates from dying populations. Compared with its influence on glycerol-accelerated death, population density had much less influence on the death-rate of glycerol- or mannitol-limited organisms starved in the presence of glucose or mannitol. Irrespective of bacterial concentration, α-ketoglutarate had no effect and pyruvate, citrate, malate, succinate and oxalacetate had less effect than glycerol on the death-rate of starved glycerol-limited bacteria.

INTRODUCTION

According to Postgate & Hunter (1963*a*, *b*; 1964), 'substrate-accelerated death' is a general phenomenon which occurs when starved bacteria are exposed to the substrate which limited their growth. Exceptions are the effects of magnesium ions and sulphate ions on magnesium- and sulphate-limited bacteria, respectively. Postgate & Hunter mention certain considerations that emphasize the practical importance of their findings, for example when bacterial death occurs because of the presence of substrate during Warburg oxygen-uptake experiments and during investigations concerned with induction or repression of enzyme synthesis. Certain possible interpretations of the phenomenon were excluded by the authors but the mechanism is not understood and deserves attention. Our work in this connexion commenced with a formal repetition of their experiments with populations of *Aerobacter aerogenes* grown at 37°. With certain important exceptions, the results with carbon- and sulphate-limited bacteria agreed with those of Postgate & Hunter but ammonium-accelerated death of ammonium-limited bacteria was not observed.

Phosphate-accelerated death apparently occurred under their conditions but the lethal effect was found to be due, not to phosphate, but to other constituents in the suspending fluid. In view of the discrepancies and the effect our findings have on the concept of substrate-accelerated death as previously reported, we record a partly repetitive investigation of the phenomenon.

METHODS

Organism. Aerobacter aerogenes (NCTC 418) was used.

Media and cultural conditions. Organisms were grown at 37° in a chemostat or in batch culture (Strange & Shon, 1964). The chemostat, designed by Dr D. Herbert, was as described by Postgate & Hunter (1962) except that the culture vessel held 250 ml. medium. All defined media contained salt mixture (Strange, Dark & Ness, 1961) (5 ml./l.), carbon energy source (2–10 g./l.), disodium ethylenediaminetetra-acetic acid (EDTA; 0·5 g./l.). Carbon-limiting medium contained (g./l.): NaH_2PO_4 (0·6), $(NH_4)_2HPO_4$ (5·95) and K_2SO_4 (1·75). Phosphate-limiting medium contained (g./l.): $NaHCO_3$ (0·42), NH_4HCO_3 (3·55), K_2SO_4 (1·75), $NaH_2PO_4.2H_2O$ (0·0625; 0·4 mM), and the pH value of the culture was maintained at 6·8–7·2 by the addition of CO_2 to the air supply. Sulphate-limiting medium contained (g./l.): NaH_2PO_4 (0·6), $(NH_4)_2HPO_4$ (5·95), KCl (0·75), Na_2SO_4 (0·0044; 0·031 mM). Nitrogen-limiting medium (1) contained (g./l.): NaH_2PO_4 (0·6), $(NH_4)_2HPO_4$ (0·99; 15 mM-N), Na_2HPO_4 (5·3), K_2SO_4 (1·75); nitrogen-limiting medium (2) contained (g./l.): $NaH_2PO_4.2H_2O$ (1·56), K_2HPO_4 (15·7), K_2SO_4 (3·5), NH_4Cl (0·268; 5 mM-N). P-, S- and N-limiting (2) media were similar to those used by Postgate & Hunter (1962) but did not contain added trace amounts of $CuCl_2$, $CoCl_2$, H_3BO_3 and Na_2MoO_4. In trial runs, the addition of these substances to media had no significant effect on the yield of bacteria, their viability (98–100 %) or their survival in the absence or presence of substrates.

Continuous cultures were run for a maximum period of 4 weeks and in no case was there a detectable change in morphology or colonial appearance of the organism. Starting inocula consisted of fully grown batch cultures of the organism in the same media (100 ml.) seeded with colonies from a stock nutrient agar plate, and shaken at 37° for 16–20 hr. Equilibrium in a chemostat was reached after 24–48 hr. Samples of cultures were examined daily for pH value, turbidity, bacterial dry weight, viable count and viability by slide culture.

Viability determinations. Viable counts were made on 3 or 5 Douglas's digest broth agar plates for each determination as previously described (Strange *et al.* 1961). The % viable bacteria in a suspension was determined microscopically with dark ground illumination after slide culture on the rich medium described by Postgate, Crumpton & Hunter (1961) except that glucose (0·2 %, w/v) replaced glycerol.

Starvation. Bacteria were separated from the culture (1 vol.) and washed in the appropriate saline buffer (2–4 vol.) by centrifugation. The bacterial pellet was gently rinsed and resuspended in saline buffer (0·1–1·0 vol.). Measured volumes of washed suspension were added to saline buffer with and without substrates (final vol., 5 or 10 ml.) usually in test tubes ($6 \times \frac{5}{8}$ in.) at 37° $\pm$ 0·1°, and aerated with sterile washed air through Pasteur pipettes. When experiments lasted longer than

6 hr, similar suspensions were agitated in Erlenmeyer flasks (50 ml.) on a reciprocal shaker at $37^\circ \pm 1^\circ$. Saline buffers with and without substrates were sterilized by filtration through well-washed sterile membrane filters (grade A.P.; Oxo Ltd.). Saline buffers were (1) 'saline tris', pH 7·0–7·2, as described by Postgate & Hunter (1962) but without EDTA; when EDTA (0·3 mM) was added this is noted in Results. Saline tris had extremely poor buffering capacity but was used for the purpose of comparing results with those of Postgate & Hunter (1962, 1963*a*, 1964). (2) 'Saline phosphate' pH 6·5 usually contained NaCl (0·11 M) and K_2HPO_4+ KH_2PO_4 (0·02 M-PO_4); in some experiments appropriate Na buffer salts replaced K salts and, in others, potassium phosphates were increased to increase buffering capacity, and NaCl was proportionally decreased. The pH values of solutions of substrates or EDTA were adjusted when necessary before addition to saline buffers.

Materials. Water used for media was passed twice through a mixed-bed ion-exchange resin (Amberlite MB-1; British Drug Houses Ltd.). Saline buffers were prepared daily with fresh glass-distilled water. Mannitol and galactose were obtained from George T. Gurr Ltd.; all other chemicals were from British Drug Houses Ltd. and, except for ribose, of Analytical Reagent grade.

Analytical methods. Protein, RNA, DNA, keto acids and bacterial dry weight were determined as previously described (Strange *et al.* 1961; Strange & Shon, 1964). Total purine pentose in hot $HClO_4$-extracts was determined by the method of Millitzer (1946). Total carbohydrate excluding pentose was determined with a modified anthrone reaction based on the method of Trevelyan & Harrison (1952), with glucose as the standard (P. J. Phipps, personal communication). Ultraviolet (u.v.) absorption spectra were obtained with an Optica recording grating spectrophotometer (model CF4DR; Optica United Kingdom Ltd., Gateshead on Tyne 11), with a 1 cm. light path.

Cell-free filtrates of bacterial suspensions were obtained by centrifugation followed by filtration of the supernatant fluids through well-washed membrane filters.

RESULTS

Properties of cultures and compositions of bacteria

Culture yields (mg. dry wt. bacteria/mg. limiting element) in chemostats and analyses of certain bacterial constituents are shown in Table 1. Comparison of these data with similar data of Postgate & Hunter (1962; their Table 4) for *Aerobacter aerogenes* (variant) growing at 40° shows that yields were similar but chemical compositions differed, sometimes markedly, at 37° and 40°. For example, N-limited organisms grown at 37°, at dilution rates of 0·18–0·61, contained 14–24 % polysaccharide as glucose, whereas those grown at 40° at a dilution rate of 0·24 contained 3·8 %, i.e. little more than C-limited bacteria grown at either temperature. P- and S-limited bacteria grown at similar dilution rates at 37° also contained considerably more polysaccharide than C-limited organisms (Table 1), but at 40°, at lower dilution rates, they contained only slightly more.

Table 1. *Effect of growth conditions on some properties of Aerobacter aerogenes populations in chemostats*

The bacteria were grown at 37° in continuous culture at different rates and with various nutrients limiting growth. Analyses refer to washed freeze-dried bacteria and usually are the mean of values obtained with several samples.

Limiting nutrient	Carbon source*	Dilution rate/hr.	Dry wt. bacteria (mg./ml. culture)	Yield†	Viable count (no./ml. culture $\times 10^{-9}$)	Bacteria: protein (%)	Bacteria: RNA (%)	Bacteria: DNA (%)	Bacteria: polysaccharide (%)
N (7·5 mM-$(NH_4)_2HPO_4$)	Mannitol (0·4%)	0·18	1·68	8	6·6	54·5	12·1	—	14·0
	Mannitol (0·6%)	0·40	1·61	7·7	3·6	51·5	18·5	—	24·0
	Mannitol (0·4%)	0·61	1·27	6·0	2·7	60·0	17·4	—	14·8
N (5 mM-NH_4Cl)	Glycerol (0·2%)	0·27	0·49	7·0	1·4	54·5	9·9	2·4	21·8
	Glycerol (0·2%)	0·39	0·48	6·85	1·6	62·0	13·8	3·2	14·7
P (0·4 mM-NaH_2PO_4)	Manitol (0·4%)	0·37	0·65	52·5	1·3	61·4	12·0	2·1	18·3
S (0·031 mM-Na_2SO_4)	Glycerol (0·2%)	0·42	0·215	209	0·2	62·3	13·5	2·5	8·4
C (21·7 mM-glycerol)	—	0·40	0·97	1·24	2·9	70·8	12·6	2·6	2·9
C (11·0 mM-mannitol)	—	0·44	1·01	1·28	3·7	71·6	14·0	—	2·6
C (11·1 mM-galactose)	—	0·39	0·89	1·11	3·9	68·0	13·8	3·0	3·8
C (13·3 mM-ribose)	—	0·37	0·84	1·05	3·8	72·0	12·7	2·7	2·7

* When different from limiting nutrient. † Mg. dry wt. bacteria/mg. element.

Effect of growth substrates on starved bacteria

Nitrogen-limited bacteria

Fourteen samples of bacteria from three chemostats, one containing N-limiting medium (1) with mannitol, and the others N-limiting medium (2) with glycerol (Table 1), were washed and starved at 37° in aerated saline tris (pH 7·0) or saline phosphate (pH 6·5) with or without NH_4Cl (10–25 mM). In no test was the survival significantly affected by NH_4Cl (Table 2). Similarly, bacteria harvested during the late-exponential and stationary phases of growth from N-limited batch cultures,

Table 2. *Effect of various substrates and EDTA on the death-rate of starved, Nitrogen-limited Aerobacter aerogenes*

Bacteria, grown in N-limiting medium (2) (dilution rate, 0·27/hr) were washed and starved (at equiv. 8 μg. dry wt. bacteria/ml.) at 37° in aerated saline tris (pH 7·0) or saline phosphate (pH 6·5), with or without addition, in shaken flasks. Viabilities (slide culture) are average of results for duplicate tests.

Diluent	Additives	Time (hr) 2	20	44
		Viability (%)		
Saline phosphate	Nil	98	98	98
	Glycerol (10 mM)	99	70	69
	Mannitol (10 mM)	98	89	65
	Glucose (10 mM)	98	63	70
	NH_4Cl (10 mM)	99	98	97
	NH_4Cl (25 mM)	98	99	93
	EDTA (0·3 mM)	97	51	*
Saline tris	Nil	99	96	98
	Glycerol (10 mM)	98	96	94
	NH_4Cl (20 mM)	98	97	97
	EDTA (0·3 mM)	99	73	57
	EDTA (0·3 mM) + NH_4Cl (20 mM)	99	97	87
	EDTA (0·3 mM) + KH_2PO_4 (10 mM)	98	50	44
	EDTA (0·3 mM) + KCl (10 mM)	97	50	43

* Bacterial lysis occurred in the suspensions.

and starved in saline phosphate, were unaffected by NH_4Cl (4·5–45 mM). These results were diametrically opposed to those of Postgate & Hunter (1963*a*, 1964) and an explanation for this discrepancy was sought. Postgate & Hunter (1962) found that EDTA (0·32 mM) improved the survival of *Aerobacter aerogenes* in saline tris at 40° and for this reason they added EDTA routinely. In the present work, numerous tests with bacteria growth-limited by N, P, S or C showed that EDTA was slightly toxic in saline tris at 37°. With N-limited bacteria, viabilities after starvation for 22 hr in saline tris with and without EDTA (0·3 mM) were 60–85 %, and 95–99 %, respectively. Since the addition of NH_4Cl (10–25 mM) to saline tris + EDTA actually improved survival, the discrepancy was not due to the absence of EDTA from our tests. Another possibility was that the bacterial concentrations used in our respective tests were sufficiently different to influence

survival in the presence of NH_4Cl. However, changing the concentration from equiv. 10 to 300 μg. dry wt. bacteria/ml. had no effect on the survival of starved N-limited bacteria exposed to NH_4Cl (20 mM).

In saline phosphate, glucose and mannitol (10 mM) increased the death rate of N-limited bacteria grown in the presence of either glycerol or mannitol as the carbon source (Table 2). Glycerol (10 mM) increased the death-rate of N-limited organisms grown in medium containing glycerol (Table 2) and the lethal effect increased with bacterial concentration up to equiv. 500 μg. dry wt./ml. In saline tris+EDTA (0·3 mM), KH_2PO_4 (10 mM) increased the death-rate of N-limited bacteria but the lethal effect was not due to phosphate because KCl (10 mM) had a similar effect (Table 2).

Table 3. *Effect of various substances on the death-rates of starved, P-limited Aerobacter aerogenes*

Bacteria grown in P-limiting medium (dilution rate, 0·37/hr) were treated as in Table 2. Pairs of viability values refer to averages of duplicate tests on different days.

Diluent	Additives	Time (hr) 2	20
		Viability (%)	
Saline tris	Nil	97, 97	95, 96
	EDTA (0·3 mM)	84, 92	51, 68
	EDTA (0·3 mM) + KH_2PO_4 (10 mM)	80, 79	37, 23
	KH_2PO_4 (10 mM)	98, 98	98, 97
	EDTA (0·3 mM) + KCl (10 mM)	88, 79	38, 22
Saline phosphate	Nil	96, 98	82, 90
	EDTA (0·3 mM)	76, 51	18, 16
Saline phosphate (Na)*	Nil	97, 97	94, 96
	EDTA (0·3 mM)	81, 84	53, 64

* Saline phosphate (Na) contained only sodium salts.

Phosphorus-limited bacteria

A formal repetition of tests described by Postgate & Hunter (1963*a*, 1964), with phosphate-limited bacteria grown at 37° in batch or chemostat cultures, apparently confirmed their findings. Thus, KH_2PO_4 (10 mM) increased the death-rate of these organisms when they were starved in saline tris containing EDTA at 37°. However, further findings (Table 3) showed that, in our experiments at least, 'phosphate-accelerated death' had not occurred. (1) When EDTA was omitted from saline tris, the survival of the bacteria was unaffected by KH_2PO_4. (2) In saline phosphate (which contained potassium phosphates) survival was good, but the addition of EDTA increased the death-rate markedly. (3) When the K phosphates in saline phosphate were replaced by Na phosphates, survival was as good in the presence of EDTA as in saline tris+EDTA. (4) In saline tris+EDTA, KCl (10 mM) increased the death-rate of the bacteria to the same extent as KH_2PO_4 (10 mM). Thus, the lethal effect of KH_2PO_4 in saline tris+EDTA was not due to phosphate ions but to the combined effect of K ions and EDTA. Also, 'phosphate-accelerated death' apparently occurred with bacteria that were not growth-limited by phosphate.

For example, N-limited bacteria growing at 37° (Table 2) and glycerol-limited bacteria growing at 40° (supplied by Mr J. R. Hunter) died more rapidly at their growth temperature in saline tris + KH_2PO_4 + EDTA than in saline tris + EDTA. In the absence of EDTA, these organisms survived well in saline tris + KH_2PO_4.

Sulphur-limited bacteria

When sulphate-limited bacteria were grown with glycerol as carbon source and starved at bacterial concentrations equiv. 10–300 μg. dry wt./ml. in saline phosphate (pH 6·5) or saline tris (pH 7·1) at 37° for 7 hr, Na_2SO_4 (10 mM) had no effect on the death-rate. In the presence of glucose or glycerol (10 mM), the viabilities of populations of these bacterial concentrations after 7 hr were 80–85 % as compared with 96–99 % in saline tris alone. These results are in agreement with those of Postgate & Hunter (1963*a*, 1964).

Carbon-limited bacteria

Glycerol-limited bacteria. Glycerol (10 mM) accelerated the death of glycerol-limited *Aerobacter aerogenes* starved in aerated saline tris or saline phosphate at 37°, but with populations equiv. 8–10 μg. bacterial dry wt./ml. the effect was considerably less than that found by Postgate & Hunter (1963*a*, 1964). Tests were therefore

Table 4. *Influence of bacterial concentration on the death-rate of Aerobacter aerogenes in the presence of glycerol*

Glycerol-limited bacteria from a chemostat (dilution rate, 0·42/hr) were washed and suspended to equiv. 8·4–1680 μg. dry wt. bacteria/ml. in aerated saline phosphate (pH 6·5) + glycerol (25 mM) at 37°. Viabilities were determined at intervals by colony counts and slide cultures.

		Colony counts		Slide culture	
		Time (min.)			
No. bacteria/ml.	Dry wt. bacteria (μg./ml.)	120	240	120	240
		% viability			
$2·8 \times 10^7$	8·4	74	73	61	58
$1·2 \times 10^8$	33·6	59	17	46	< 1
$5·5 \times 10^8$	168	< 1	< 1	< 1	< 1
$5·5 \times 10^9$	1680	100*	100*	91	90

* Colony count 7–9 % higher than starting value.

repeated at 40° with *A. aerogenes* growing at 40° (supplied by Mr J. R. Hunter). In saline phosphate, glycerol accelerated death to a greater extent than with bacteria grown and starved at 37° but the effect was still less than that observed by Postgate & Hunter; in saline tris + EDTA, glycerol had a marginal effect. This difference of results was probably partly due to the difference in bacterial concentrations routinely used in the tests by Postgate & Hunter (equiv. 20 μg. dry wt./ml.) and ourselves (equiv. 8–10 μg. dry wt./ml.); between equiv. 8 and about 800 μg. bacterial dry wt./ml., glycerol-accelerated death increased (not decreased as reported by Postgate & Hunter, 1964) with bacterial concentration (Fig. 1). With denser populations (above equiv. 1000 μg. dry wt. bacteria/ml.), substrate utilization produced sufficient acid to change significantly the pH value of saline

phosphate. The pH value of dense suspensions of bacteria was maintained by using a modified saline phosphate (0·03 M-NaCl and 0·06 M-phosphate, pH 6·5) or by adding dilute NaOH at intervals to suspensions in normal saline phosphate. Under these conditions the death-rate in the presence of glycerol decreased with increasing bacterial concentration above equiv. about 800 μg. dry wt./ml. The influence of bacterial concentration on glycerol-accelerated death was also seen when viabilities were determined by colony counts as well as slide culture (Table 4). The phenomenon was seen with two different samples of A.R. glycerol and a third sample obtained from one of these by distillation under reduced pressure. Changing the concentration of glycerol from 10 to 100 mM in the tests also had no effect on the death-rates, so that it can be assumed that the results were not due to the use of impure glycerol.

Bacteria-free filtrates from suspensions (equiv. 15, 150 and 1500 μg. dry wt. bacteria/ml.) incubated in the presence of glycerol had a lethal effect on fresh

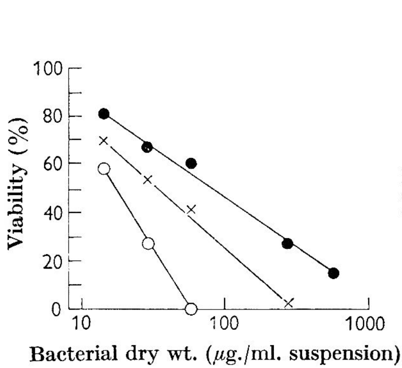

Fig. 1

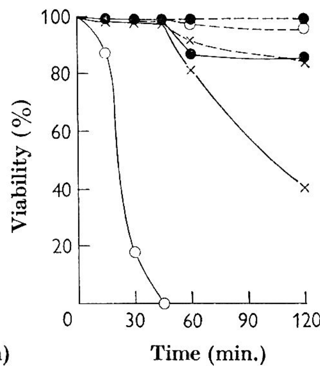

Fig. 2

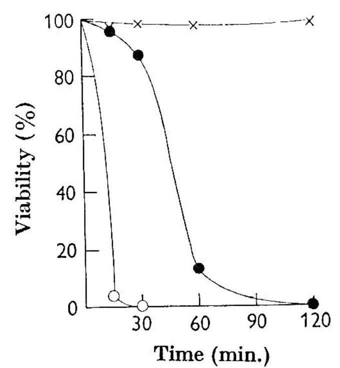

Fig. 3

Fig. 1. Influence of bacterial concentration on the death-rate of *Aerobacter aerogenes* in the presence of glycerol. Glycerol-limited bacteria from a chemostat (dilution rate, 0·42/hr) were washed and suspended at concentrations equiv. 14·5–5800 μg. dry wt. bacteria/ml. in aerated saline phosphate, with and without glycerol (12·5 mM) at 37°. Viabilities (slide culture) of the suspensions after 1 hr (●), 2 hr (×) and 4·5 hr (○). Viabilities of all suspensions without glycerol were 95–98 % and suspensions equiv. 2900 and 5800 μg. dry wt. bacteria/ml. with glycerol were 93 and 96 %, respectively, after 4·5 hr at 37°. Ordinate scale (viability) is arithmetic and abscissa scale (dry wt.) logarithmic.

Fig. 2. Lethal effect of filtrates from suspensions of *Aerobacter aerogenes* dying in the presence of glycerol. Glycerol-limited bacteria (as in Fig. 1) at concentrations equiv. 15, 150 and 1500 μg. dry wt. bacteria/ml. in saline phosphate, with and without glycerol (10 mM), were aerated at 37° for 90 min. Viabilities were 71, 1 and 63 %, respectively, in presence of glycerol, and 96, 97, 96, respectively, in its absence. Bacteria-free filtrates of the six suspensions were prepared and incubated with fresh bacteria (equiv. 9·5 μg. dry wt./ml.) in the presence of added glycerol (10 mM). Viabilities of populations in filtrates from bacterial suspensions equiv. 15 (×), 150 (○) and 1500 (●) μg. dry wt. bacteria/ml. Filtrates from suspensions with glycerol, unbroken lines; without glycerol, broken lines.

Fig. 3. Effect of dialysis on glycerol-accelerated death of *Aerobacter aerogenes*. Glycerol-limited bacteria (as in Fig. 1) equiv. 200 μg. dry wt./ml. in aerated saline phosphate (5 ml.) + glycerol (10 mM) were dialysed at 37° in a cellophan sac against 200 ml. diluent + glycerol which was changed every 30 min. A control suspension without glycerol was similarly dialysed against diluent alone, and a third suspension with glycerol was aerated at 37° without dialysis. Suspension dialysed in the presence of glycerol, ●; dialysed without glycerol, ×; undialysed suspension with glycerol, ○.

bacteria; filtrate from the suspension equiv. 150 μg. dry wt./ml. (in which the fastest death-rate occurred) was the most lethal (Fig. 2). Filtrates from similar control suspensions (incubated without glycerol) were non-toxic and filtrate from the strongest bacterial suspension partially protected fresh bacteria from the lethal effect of added glycerol. Dialysis against phosphate saline + glycerol at 37° of an aerated bacterial suspension (equiv. 200 μg. dry wt./ml.) containing glycerol markedly decreased the death-rate as compared with that in a similar undialysed suspension (Fig. 3). These experiments show that the population density phenomenon in the presence of glycerol was due to the formation of both toxic and protective substances by the bacteria; the toxic substance diffused through the cellophan in dialysis.

Table 5. *Effect of magnesium on glycerol-accelerated death and the toxicity of filtrates from suspensions of Aerobacter aerogenes dying in the presence of glycerol*

Bacteria grown in glycerol-limiting medium (dilution rate, 0·41/hr) were washed and suspended to equiv. 16 μg. dry wt. bacteria/ml. in saline phosphate (pH 6·5) and in bacteria-free filtrate (of a suspension equiv. 160 μg. dry wt. bacteria/ml. + glycerol (10 mM) aerated at 37° for 2 hr; viability < 1 %) + glycerol (10 mM), with and without Mg^{2+} (mM). Viabilities (slide culture) were determined at intervals.

Diluent	Mg^{2+} (mM)	Time (min.) 15	30	60	90
		Viabilities (%)			
Saline phosphate	−	98	83	59	49
	+	100	98	96	98
Filtrate	−	79	19	5	< 1
	+	93	34	.	11

Table 6. *Effect of rate of bacterial oxidation of glycerol on glycerol-accelerated death*

Aerobacter aerogenes grown in defined C-limiting medium in batch or continuous cultures, washed and suspended in saline phosphate (pH 6·5); Q_{O_2} (glycerol) values were determined in the Warburg apparatus at 37°. Suspensions equiv. 10–800 μg. dry wt. bacteria/ml. were aerated at 37° with glycerol (20 mM) and the viabilities (slide culture) determined after 4 hr.

Culture conditions	Bacterial Q_{O_2} (glycerol)	Dry wt. bacteria (μg./ml.) 10	200	400	800
		Viability (%) after 4 hr			
Mannitol-limiting medium in shaken flasks	20	99	99	99	99
Mannitol-limiting medium with 0·02 % (w/v) glycerol in chemostat	142	84	61	49	84
Glycerol-limiting medium in chemostat	340	28	< 1	< 1	1

As found by Postgate & Hunter (1964), Mg^{2+} abolished glycerol-accelerated death; Mg^{2+} had less effect on the toxicity of bacteria-free filtrates from suspensions dying in the presence of glycerol (Table 5).

Glycerol-accelerated death also depended on the rate at which glycerol was oxidized by the bacteria. Table 6 compares the death-rates of similar concentrations of three batches of bacteria, with different Q_{O_2} (glycerol) values, in saline phosphate + glycerol. Bacteria with the highest Q_{O_2} value died at the fastest rate.

Glucose and mannitol also accelerated the death of starved glycerol-limited bacteria but, up to equiv. 800 μg. dry wt. bacteria/ml., population density had little effect on the death-rate; for example, the viabilities of suspensions containing equiv. 10, 200, 400 and 800 μg. dry wt. bacteria/ml. with glucose (20 mM) after 280 min. at 37° were 46, 42, 47 and 50 %, respectively. Malate, pyruvate, succinate, citrate and oxalacetate had small effects, compared with glycerol, on the death-rate (Table 7). α-Ketoglutarate had no effect although Postgate & Hunter (1964) found that this keto acid accelerated the death of their bacteria.

Table 7. *Effect of metabolic intermediates on the death-rate of starved Aerobacter aerogenes*

Bacteria as in Table 4 were starved at concentrations equiv. 20 and 200 μg. dry wt. bacteria/ml. in aerated phosphate saline (pH 6·5) containing addition (10 mM) at 37°. Viabilities were determined after 120 min. (slide culture).

Addition to diluent	Dry wt. bacteria (μg./ml.) 20	200
	Viability (%)	
Glycerol	38	1
α-Ketoglutarate	97	95
Citrate	79	99
Malate	90	88
Succinate	88	87
Oxaloacetate	90	94
Pyruvate	52	90

Table 8. *Influence of bacterial concentration on the death-rate of Aerobacter aerogenes in the presence of mannitol*

Mannitol-limited bacteria from a chemostat (dilution rate 0·44/hr) were washed and suspended at equiv. 5–800 μg. dry wt. bacteria/ml. in aerated phosphate saline (pH 6·5) + mannitol (10 mM) at 37°. Viabilities (slide culture) were determined at intervals.

Dry wt. bacteria (μg./ml.)	Time (hr) 1	2	4	6
	Viability (%)			
5	55	45	53	49
100	66	59	60	56
400	63	58	53	52
800	85	69	57	50

Mannitol-limited bacteria. Glucose, mannitol, and, to a smaller extent, galactose and ribose, increased the death-rate of starved mannitol-limited *Aerobacter aerogenes*. Glycerol had no effect on bacteria with a low Q_{O_2} (glycerol) value (Table 6). The effect of bacterial concentration (equiv. 5–800 μg. dry wt./ml.) on mannitol- and glucose-accelerated death of these bacteria was not very great (Table 8). The survival of fresh bacteria in filtrates from populations of different concentrations

dying after 6 hr in the presence of mannitol varied; viabilities of bacteria (equiv. 10 μg. dry wt./ml.) after 2 hr at 37° in aerated phosphate saline, phosphate saline + mannitol (10 mM) and filtrates from suspensions equiv. 5, 100, 400 and 800 μg. dry wt. bacteria/ml. were 92·5, 55, 62·5, 41·2, 5·6 and 36·2 %, respectively.

Galactose- and ribose-limited bacteria. The effects of carbon substrates on the viability of suspensions equiv. 10 μg. dry wt. galactose- or ribose-limited bacteria/ml. starved in phosphate saline at 37° are shown in Table 9. Substrates other than those which limited growth affected the death-rates, but glycerol, which was oxidized at a slow rate (Q_{O_2}, 17–24), had little effect.

Table 9. *Effect of carbon substrates on the death-rates of starved galactose- and ribose-limited Aerobacter aerogenes*

Bacteria grown in galactose- or ribose-limiting defined media (dilution rate, 0·41/hr) were washed and suspended (at equiv. 10 μg. dry wt. bacteria/ml.) in saline phosphate (pH 6·5) with and without substrates (10 mM) and shaken at 37°. Viabilities (slide culture) were determined at intervals on duplicate tests.

Growth-limiting substrate	Suspension substrates (10 mM)	Bacterial Q_{O_2} with substrate	Time (hr) 2	4	20	44
			Viability (%)			
Galactose	Nil	—	98	98	95	94
	Galactose	210	92	85	30	29
	Glucose	197	93	90	59	47
	Glycerol	24	96	98	92	80
Ribose	Nil	—	100	97	94	89
	Ribose	230	96	92	61	38
	Galactose	89	96	92	65	35
	Glucose	144	93	90	69	48
	Glycerol	17	98	98	99	90

Biochemical changes occurring in suspensions of Aerobacter aerogenes starved in the presences of carbon substrates

Filtrates from suspensions of C-limited bacteria dying in the presence of glucose, mannitol or glycerol were yellow; they absorbed in the ultraviolet (u.v.) region strongly and, as found by Postgate & Hunter (1964) in the case of glycerol-accelerated death, these filtrates contained material with a maximum absorption at about 210–240 mμ. Keto acids determined by the method of Friedmann & Haugen (1943) were present in filtrates from suspensions of bacteria incubated with mannitol or glycerol; paper chromatographic examination (El Hawary & Thompson, 1953) showed that these were mainly accounted for as α-ketoglutaric acid. The presence of this keto acid was confirmed spectrophotometrically with glutamic acid dehydrogenase. Comparison of the u.v. absorption of α-ketoglutarate and filtrates (Fig. 4) suggests that the strong absorption of the filtrates at 210–240 mμ was due to keto acids. However, the toxicity of filtrates from organisms dying in the presence of substrate was not due to α-ketoglurate because this keto acid had no effect on the death-rate of starved glycerol- (Table 7) or mannitol-limited bacteria, nor did it increase the lethal effect of glycerol on glycerol-limited bacteria. Magnesium abolished substrate-accelerated death but not the release of α-ketoglutarate from mannitol-limited bacteria starved in the presence of mannitol, and in the presence

Table 10. *RNA degradation in suspensions of Aerobacter aerogenes starved in the presence of carbon substrates*

Mannitol-limited bacteria (as in Table 8) were starved at concentration equiv. 500 μg. dry wt. bacteria/ml.) in aerated saline phosphate (pH 6·5), with and without substrates (25 mM) at 37°. Samples removed at intervals were assayed for viability and RNA.

	Time (min)					
	45	75	165	45	75	165
Substrate	Viability (%)			RNA (% initial value)		
Nil	98	99	98	98	99	88
Mannitol	87	65	57	92	82	78
Glucose	83	61	50	89	84	77
Glycerol	99	98	97	99	92	92

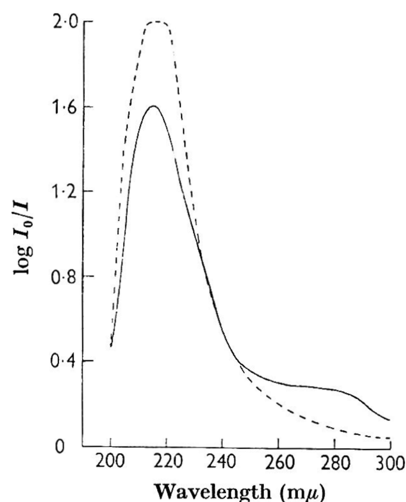

Fig. 4

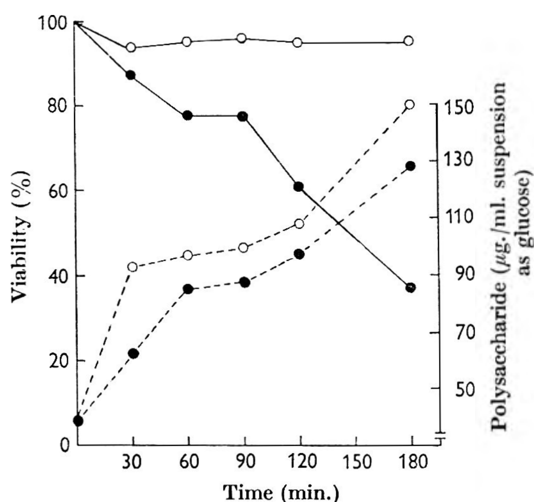

Fig. 5

Fig. 4. Comparison of the u.v.-absorption of α-ketoglutarate, and filtrate from *Aerobacter aerogenes* dying in the presence of glycerol. Cell-free filtrate was prepared from a suspension equiv. 500 μg. dry wt. bacteria/ml. saline phosphate + glycerol (10 mM) which had been aerated at 37° for 3 hr and contained 242 μg. keto acid as α-ketoglutaric acid/ml. Filtrate diluted 1/3 in saline phosphate (unbroken line); α-ketoglutarate (mM) in saline phosphate (broken line).

Fig. 5. Effect of magnesium on the death-rate and polysaccharide synthesis in *Aerobacter aerogenes* starved in the presence of mannitol. Mannitol-limited bacteria from a chemostat (dilution, 0·4/hr) were washed and starved at concentration (equiv. 1000 μg. dry wt. bacteria/ml.) in aerated saline phosphate (pH 6·5) + glycerol (25 mM), with and without Mg^{2+} (mM), at 37°. At intervals samples (2 ml.) were centrifuged and the bacteria resuspended in water (1·5 ml.) for polysaccharide determinations. Viability (slide culture), unbroken lines; polysaccharide (μg./ml.), broken lines. Suspension with Mg^{2+}, ○; without Mg^{2+}, ●.

of Mg^{2+} the synthesis of reserve polysaccharide was marginally increased (Fig. 5). In the absence of added magnesium, the rate of RNA degradation in these bacteria in the presence of mannitol was significantly greater than in similar bacteria starved in phosphate saline, with or without glycerol (Table 10).

DISCUSSION

The present results do not support the view of Postgate & Hunter (1964) that 'substrate-accelerated death' is ubiquitous; the results restrict the phenomenon to the lethal effect of carbon energy sources metabolized by *Aerobacter aerogenes* in the absence of added magnesium and other nutrients. Differences in the polysaccharide content of the bacteria, and/or the growth and starvation conditions, may account for our failure to observe ammonium-accelerated death of ammonium-limited bacteria. But it is evident that the phenomenon is less general than was previously supposed. The lethal effect of potassium phosphate on phosphate-limited bacteria starved in the presence of EDTA is confirmed but the previous interpretation of this result as 'phosphate-accelerated death' is shown to be incorrect.

Carbon substrate-accelerated death of *Aerobacter aerogenes* is confirmed but a specific lethal effect of the growth-limiting carbon source was not evident, although, where metabolic pathways for a particular substrate (e.g. glycerol) involved induced enzyme formation, the growth-limiting substrate might have a greater effect than other substrates. Glucose increased the death-rate of starved bacteria irrespective of the growth-limiting substrate. To some extent this result was anticipated since it had been shown previously that this substrate accelerated the death of *A. aerogenes* grown in a complex medium (Strange *et al.* 1961). Glycerol-accelerated death of bacteria fully adapted to metabolize glycerol differed from glucose- or mannitol-accelerated death with respect to the influence of the concentration of bacteria on the lethal effect of the substrate. The effect with glycerol, where below a certain bacterial concentration the death-rate increased with the concentration, is due to the production of a product(s) from glycerol which must reach a threshold concentration in the suspending fluid before toxicity is evident. This product has not been identified and it is not possible to say whether the protective effect of bacterial concentration above the critical value is due to restricted synthesis or utilization of the product, or to the neutralizing effect of other substances released from the bacteria. Magnesium abolished glycerol-accelerated death, irrespective of population density, but only marginally decreased the lethal effect of filtrate from bacteria dying in the presence of glycerol. This suggests that magnesium prevents accumulation of the toxic product, either by inhibiting its synthesis or by stimulating its utilization. Although the influence of bacterial concentration on mannitol- (or glucose-) and glycerol-accelerated death was different, the different effects of filtrates from different concentrations of bacteria dying in the presence of mannitol on the death-rate of fresh bacteria suggests that, here too, toxic and protective factors were involved. The present findings do not provide an explanation of the mechanism of substrate-accelerated death but evidence that the metabolism of glycerol, and possibly of other carbon substrates, in the absence of other nutrients and/or magnesium, leads to an accumulation of toxic products which account for perhaps all of the lethal effect, may help further investigation.

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Factors which Affect the Release of Newcastle Disease and Sendai Viruses from Infected Allantoic Cells

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SUMMARY

Some aspects of the adsorption and release of Newcastle disease and Sendai viruses were studied in excised pieces of allantois of uniform size. About 40 % of any saturating dose of either virus was adsorbed. The % adsorption was not influenced by treatment with neuraminidase. One-step growth curves indicated that the release process for both viruses was linear. The rate of release and the burst size were influenced by the composition of the medium used for maintaining the allantoic cells. Increases in the multiplicity of infection decreased the length of the latent period. Treatment of allantoic cells with suitable doses of actinomycin D or ultraviolet irradiation also affected the length of the latent period. These agents may increase the susceptibility of cells to infection by affecting DNA-directed mechanisms concerned in the control of nucleic acid synthesis.

INTRODUCTION

Most myxoviruses grow well in the allantoic cells of the developing chick embryo. However, detailed quantitative studies have been made only with influenza virus. It has gradually become apparent that the myxoviruses are not a homogeneous group of closely related viruses, but consist of two distinct varieties (Waterson, 1962), of which the influenza viruses constitute one type. Newcastle disease virus (NDV) and Sendai virus are representatives of the other type of myxovirus, and it is the purpose of this paper to examine some quantitative aspects of their multiplication in allantoic cells.

METHODS

Strains of virus. The following strains of virus were used: NDV California 11914, and Sendai (originally obtained from The National Institute for Medical Research, Mill Hill, through the kindness of Dr A. Isaacs). Each strain consisted of an allantoic fluid material prepared and stored as previously described for influenza virus stocks (Barry, 1961).

Neuraminidase. Preparations of this enzyme were obtained from Behringwerke, Marburg, Germany. Titrated by the method of Burnet & Stone (1947), they contained 10^4 units of activity/ml.

Haemagglutination titrations. Haemagglutination (HA) titrations were done by the method of Fazekas de St Groth & Graham (1955), except that 0·25 ml. volumes of a 0·5 % (v/v) fowl red cell suspension were used. When HA titrations were made in the presence of neuraminidase, trays were chilled to 2° before the addition of cells.

Media and solutions. PBS: phosphate-buffered saline (Dulbecco & Vogt, 1954). SM: 'Standard Medium', a balanced salt solution (Fazekas de St Groth & White, 1958) used for the maintenance of allantoic cells. 199: Parker's chemically defined medium 199 (Morgan, Morton & Parker, 1950) was obtained from Burroughs Wellcome and Company, London.

Infectivity titrations. Infectivity was titrated by the method of Fazekas de St Groth & White (1958), except that medium 199 was substituted for SM in the maintenance of membrane pieces.

The experimental system. Experiments were made with pieces of allantois-on-shell, 1 cm.^{2} in size, obtained from 11-day or 12-day chick embryos. Barry (1961) described in detail the preparation and handling of such pieces. The pieces were placed individually in cups of large Perspex trays. Each cup contained 0·7 ml. of the appropriate medium, and virus was added in 0·02 ml. drops. Trays were then incubated on a shaking machine in a constant temperature room at 35°. Measurements of the uptake of virus, or of the yield of virus were determined by HA titration. Except where otherwise stated, each experimental point represents the average value obtained from four replicate pieces. For any particular experimental conditions, the yields of individual pieces showed little variation, e.g. 24 pieces, incubated in SM and infected with a saturating dose of NDV, produced an average yield 18 hr later of $2^{5.4 \pm 0.2}$. The range of individual values was from $2^{5.2}$ to $2^{5.7}$.

Actinomycin. The actinomycin D was kindly provided by Dr J. Merry (Merck, Sharp and Dohme Ltd., Hoddesdon, Hertfordshire). Solutions containing 100 μg./ml. were prepared in distilled water and stored frozen. Suitable dilutions were prepared in SM or 199 media and then applied to cells.

Ultraviolet irradiation. Allantoic cell pieces washed several times in warm PBS were exposed for 10 sec., in open Petri dishes, to a 'chromatolite' portable ultraviolet lamp (Hanovia Ltd.), at a distance of 25 cm. More than 95 % of the output of this lamp had a wavelength 2537 Å.

RESULTS

Estimation of multiplicity

The adsorption of myxoviruses to host cells usually requires mucoproteins. In the case of influenza virus however, much of the virus attached to surface mucoproteins does not penetrate inside the host cells. To overcome this difficulty when measuring the multiplicity of infection, Cairns & Edney (1952) treated cells, after exposure to virus, with neuraminidase, which releases all superficially bound virus. Under these conditions the actual amount of virus irreversibly bound by cells was always about 50 % of the input. Before proceeding with studies of the growth cycle of NDV and Sendai, it was necessary to find how much virus was irreversibly bound to cells under different conditions of input. The % adsorption of these viruses to allantoic cells in the absence and presence of neuraminidase was, therefore, determined.

A dilution series of NDV allantoic fluid was prepared, and each dilution was added to batches of five replicate allantoic pieces. The membrane pieces were incubated for 90 min. on the shaking machine, after which time the uptake of virus to cells was measured by determining the HA content of the supernatant medium

(Fig. 1). Regardless of the actual amount of virus in the inoculum, about 40 % of it became associated with the cells. In a similar experiment, identical results were obtained for Sendai virus.

To determine whether the % adsorption of either virus was influenced by treatment with neuraminidase, batches of allantoic pieces were infected with various inputs of virus, and adsorption allowed to proceed for 90 min. when each batch was

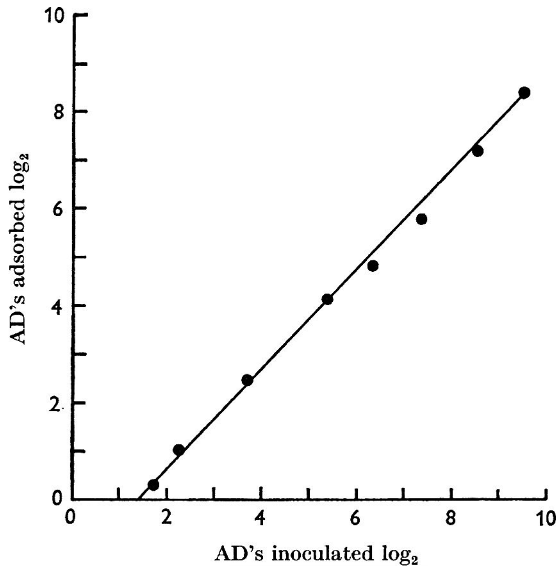

Fig. 1. The relation between the amount of virus inoculated and the amount adsorbed by pieces of allantois. One agglutinating dose (AD) of virus is equivalent to $10^{6\cdot3}$ ID50.

Table 1. *The effect of neuraminidase on the percentage adsorption of Sendai virus to allantoic cells*

Input no.	HA content/0·25 ml. supernatant fluid ($\log_2$)			% adsorption	
	Controls	Treated*	Untreated	Treated*	Untreated
1	6·3	5·4	5·5	46	40
2	4·7	3·9	4·0	42	38
3	3·5	2·8	2·6	38	46
4	2·2	1·5	1·5	39	39

* Ninety minutes after addition of virus, treated allantoic cell pieces were exposed to 100 units neuraminidase for a further 60 min.

divided into two. Half of the pieces were treated with 100 units neuraminidase for 1 hr while the remainder were not. Controls consisted of 0·7 ml. volumes of medium containing virus but not allantoic pieces. At 2 hr after infection, the uptake of virus was determined by HA titration. The amount of Sendai virus irreversibly bound to host cells was uninfluenced by neuraminidase, and was about 40 % of the total inoculum (Table 1). NDV behaved si ilarly.

Each allantoic piece consists of about $3\cdot3 \times 10^5$ surface allantoic cells (Cairns &

Fazekas de St Groth, 1957). The stocks of NDV and Sendai were fully infectious; i.e. the ID50/HA ratio of each was $10^{6\cdot3}$. The virus content of any inoculum was corrected to infectious units (IU) by subtraction of 0·15 $\log_{10}$ from its total ID50 content. A further 0·4 $\log_{10}$ was subtracted from the total ID50 content to allow for that proportion of the input not adsorbed. It was thus possible to obtain an estimate for the maximum adsorbed multiplicity of infectious units per cell for any input of virus.

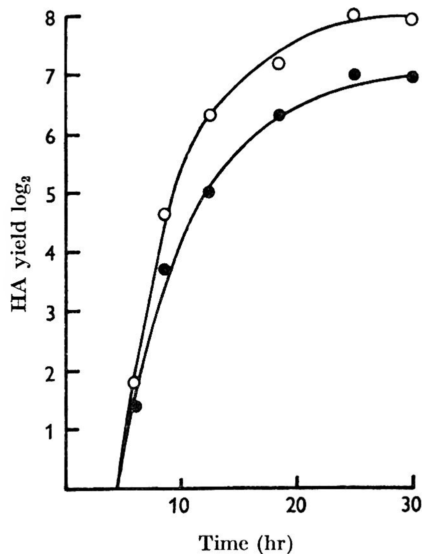

Fig. 2

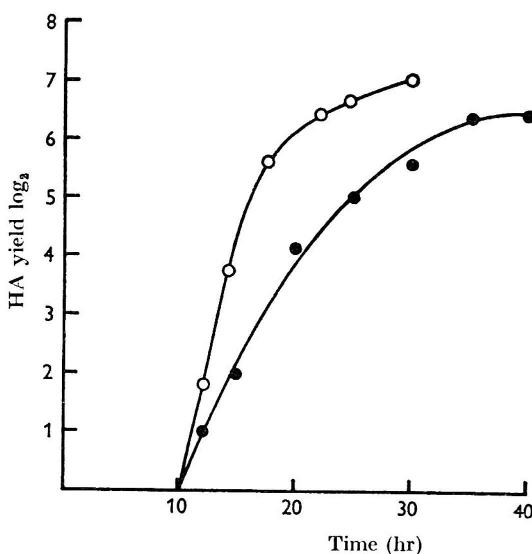

Fig. 3

Fig. 2. One-step growth curves for NDV in allantoic cells. Cells were infected with an estimated multiplicity of 4 IU per cell. ○ = yields in 199; ● = yields in SM.

Fig. 3. One-step growth curves for Sendai virus in allantoic cells. Cells were infected with an estimated multiplicity of 4 IU per cell. ○ = yields in 199; ● = yields in SM.

One-step growth curves

The growth of NDV and Sendai viruses was investigated by obtaining one-step growth curves. Experiments were performed by infecting allantoic pieces for 90 min. with saturating doses of known virus content. After removal of the inoculum the pieces were washed in several changes of warm PBS. Fresh medium was then added to each piece and the trays replaced on the shaker. Subsequently, batches of infected pieces were removed at intervals and the medium in which they had been incubated was assayed for haemagglutinin. The cells received an input of virus estimated to provide an adsorbed multiplicity of 4 IU per cell. The results indicated that for both viruses the maintenance medium of the host cells influenced release rate and burst size (Figs. 2, 3). Under optimal conditions, each membrane piece is capable of producing about 200 HA units of NDV, and half this amount of Sendai. Assuming an ID50/HA ratio of about $10^{6\cdot3}$, each cell is then capable of producing approximately 1000 ID50 of NDV, or 500 ID50 of Sendai virus.

The effect of multiplicity of infection

Although individual allantoic cells infected with influenza virus release the bulk of their yield over a short period of time, considerable variation exists between cells in the time of onset of this process (Cairns, 1957). This variation can be decreased by increasing the absorbed multiplicity of infection per cell. In a one-step growth experiment, reduction in asynchrony increases the rate of release. However, increasing the multiplicity of infection does not affect the time of onset of the

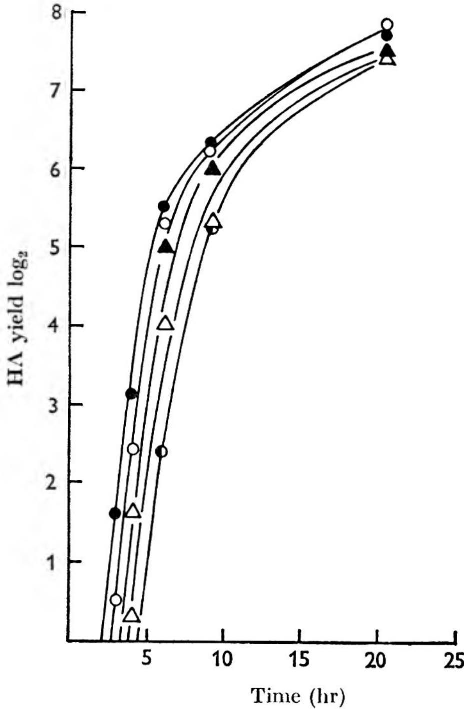

Fig. 4

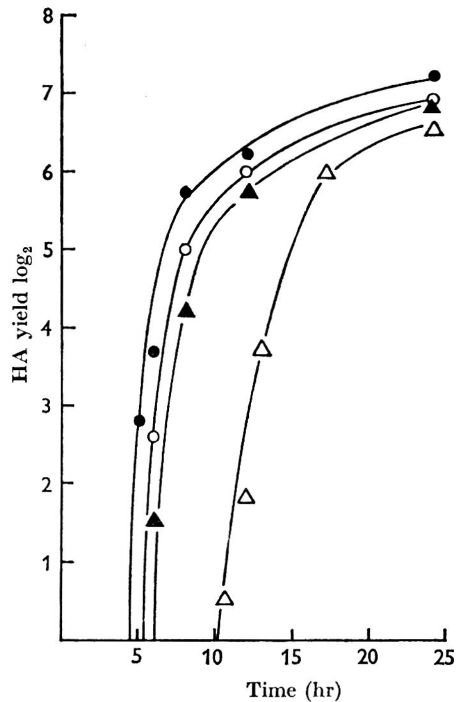

Fig. 5

Fig. 4. The effect of adsorbed multiplicity on the length of the latent period of NDV multiplication. From left to right, estimated adsorbed multiplicities of infection of approximately 500, 120, 30, 8 and 2 IU per cell, respectively.

Fig. 5. The effect of adsorbed multiplicity on the length of the latent period of Sendai virus multiplication. From left to right, estimated adsorbed multiplicities of infection of, approximately, 1000, 500, 120 and 10 IU per cell, respectively.

release process. To determine whether the release process of NDV or Sendai is similar to that of influenza virus, one-step growth curves were made with different multiplicities of infection. Five batches of allantoic pieces were prepared and incubated in medium 199. Each batch received a particular dose of NDV such that, after adsorption, approximate multiplicities of 500, 120, 30, 8 and 2 IU per cell were achieved. The experiment was incubated at 35° on the shaker, and at suitable intervals after infection the HA yield of groups of pieces from each batch was measured. As the multiplicity of infection increased, the time of appearance of

newly formed virus shortened, but the rate of virus release was not affected (Fig. 4). Consequently, the response of cells to increasing doses of NDV differs from that found with influenza virus.

The latent period of Sendai virus multiplication was also influenced by the adsorbed multiplicity of infection. Batches of allantoic pieces were infected with estimated multiplicities of 1000, 500, 120 and 10 IU of Sendai virus per cell, and growth curves for each input were obtained. Increased multiplicity of infection clearly decreased the latent period (Fig. 5).

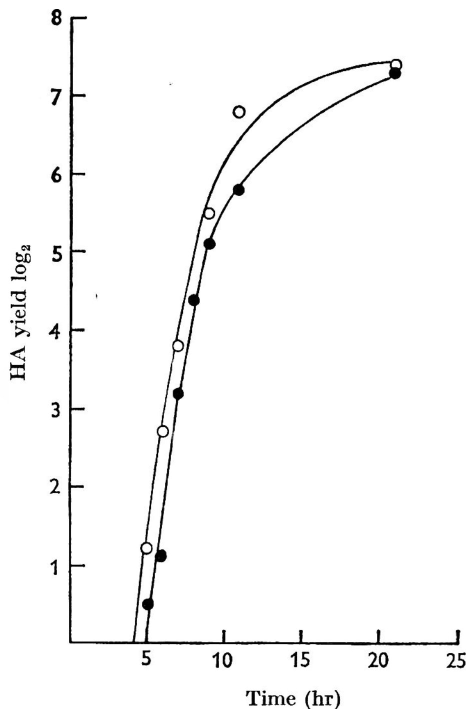

Fig. 6

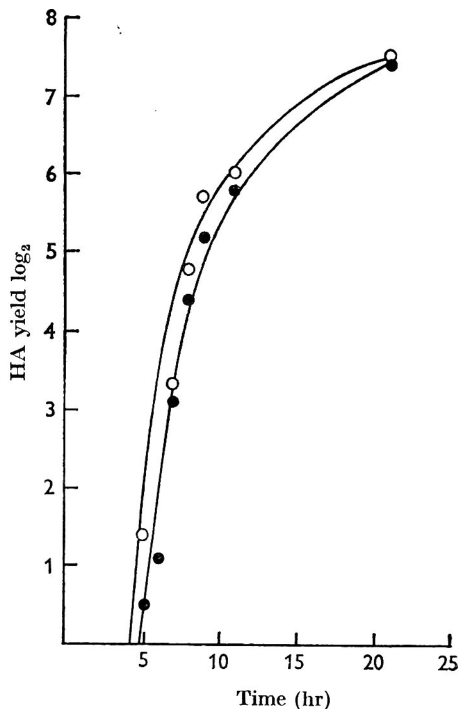

Fig. 7

Fig. 6. The effect of ultraviolet irradiation on the release of NDV. Allantoic cells were subjected to 10 sec. u.v. irradiation before infection with NDV at multiplicity of 2 IU per cell. ○ = yields from irradiated cells; ● = yields from unirradiated control cells.

Fig. 7. The effects of actinomycin D 1 μg./ml. on the release of NDV. ○ = yields from treated cells infected with 2 IU per cell; ● = yields from untreated cells infected with 2 IU per cell.

The effects of inhibitors of nucleic acid synthesis

The multiplication of influenza virus is suppressed by actinomycin D and by ultraviolet (u.v.) irradiation. These agents affect the function of cellular DNA (Barry, Ives & Cruickshank, 1962; Barry, 1964). Doses of either inhibitor which decrease the yield of influenza by 99% do not affect the yield of either NDV or Sendai. In preliminary studies, it was found that treatment of allantoic cells with small doses of either inhibitor (1–2 μg./ml. actinomycin, up to 10 sec. of u.v. irradiation) apparently increased the yield of NDV. The nature of this effect was in-

vestigated further by examining the effect of apparently stimulating doses of u.v. radiation or actinomycin D on the growth cycle of NDV and Sendai. One-step growth curves were obtained for both viruses in the presence and absence of stimulating doses of each agent. One batch of allantoic pieces was subjected to 10 sec. of u.v. irradiation before exposure to a dose of NDV giving an absorbed multiplicity of 2 IU/cell. The results were compared to those obtained from infected, unirradiated control pieces. This dose of u.v. radiation did not influence the size of the final yield or the release rate, but did influence the time of onset of virus release (Fig. 6).

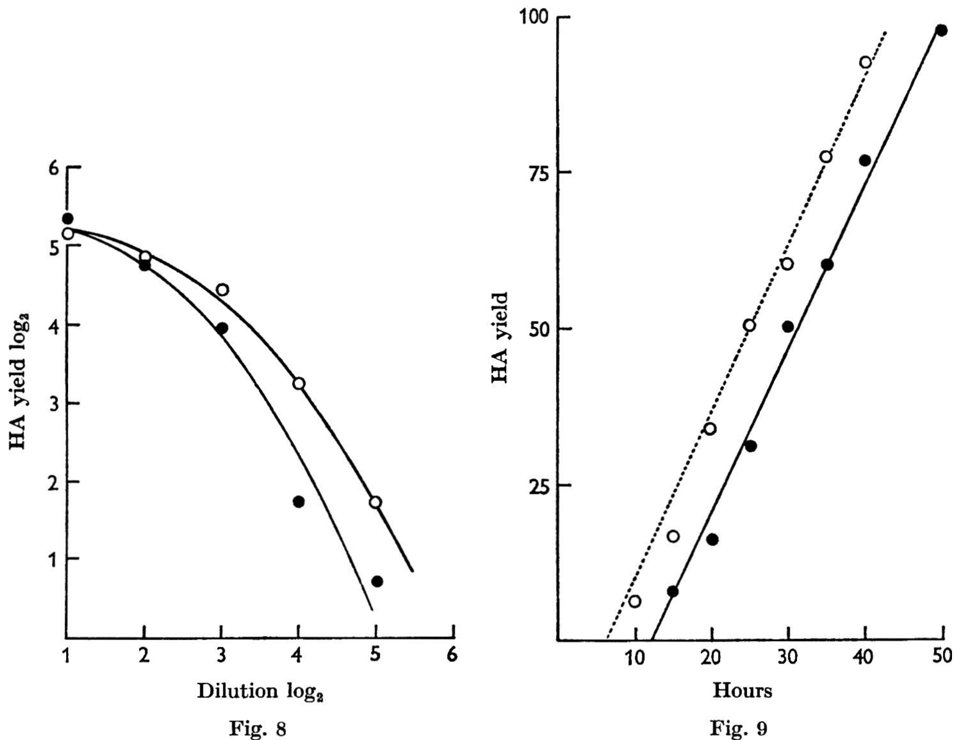

Fig. 8. The effects of actinomycin D 1 μg./ml. on the amount of Sendai virus released from allantoic cells 10 hr after infection with a range of input multiplicities. ○ = yields from treated cells; ● = yields from untreated cells.

Fig. 9. The linearity of the release process of Sendai virus from allantoic cells when infected with 10 IU per cell. ○ = yields in the presence of actinomycin D 1 μg./ml.; ● = yields in the absence of actinomycin D.

Identical results were obtained with Sendai virus. Suitable concentrations of actinomycin also influenced the release rate. Cells were again infected at a multiplicity of 2 NDV IU/cell. Half the pieces were incubated in actinomycin D (1 μg./ml.) The results were the same as those obtained with u.v. radiation (Fig. 7), indicating that this substance at this concentration also shortened the latent period of infection. Similar results were obtained with Sendai virus. Much higher doses of either u.v. radiation or actinomycin D did not affect the total yield of NDV or Sendai, but the latent period is influenced only when small doses are used.

To demonstrate that inhibitor-induced shortening of the latent period occurred

at a variety of input multiplicities, batches of allantoic pieces were infected with individual dilutions of Sendai virus, providing adsorbed multiplicities of 160, 80, 40, 20 and 10 IU per cell. Each batch was divided in two, one half of the pieces receiving 1 μg./ml. actinomycin D, the remaining pieces serving as controls. Infection was allowed to proceed for 10 hr and then HA yields were determined. The release of newly formed virus occurred more rapidly from cells treated with actinomycin D than from the controls, although, as the multiplicity of infection increased, this effect was gradually obscured (Fig. 8).

The susceptibility of host cells

Suitable doses of actinomycin D or u.v. radiation make host cells respond to infection as though they had received a higher input of virus. In the examples shown in Figs. 6 and 7, the cells responded as though they received an input four times higher than that actually used. To determine whether this effect was attributable to an increase in the susceptibility of host cells to infection, each virus was titrated for infectivity in cells subjected to 10 sec. of u.v. irradiation, and the titres compared to those obtained in untreated cells. In those experiments, twofold dilutions of virus were prepared and 0·02 ml. volumes of each were inoculated into each of 50 allantoic pieces. Treatment of cells before infection with a stimulating dose of u.v. radiation resulted in an approximately twofold increase in the end-points obtained for either virus (Table 2).

Table 2. *The susceptibility of ultraviolet-irradiated allantoic pieces to infection*

	Infectivity titre/0·25 ml. ($\log_{10}$)	
Virus	Control	*U.v. irradiated
NDV	8·60	8·95
Sendai	8·35	8·63

* Pieces of allantois were exposed in open Petri dishes to irradiation at wavelength 2537 Å for 10 sec. at a distance of 25 cm.

DISCUSSION

The presence of adsorbed virus at the surface of host cells, and its subsequent elution, was an unexpected complication in some quantitative studies of influenza virus multiplication (Cairns, 1955), but could be avoided when the cells were pretreated with neuraminidase after adsorption (Cairns & Edney, 1952). In the present study, it was found that, unlike influenza, the % adsorption of any saturating dose of either NDV or Sendai virus was not complicated in this way. Only 40 % of the total input was irreversibly bound by allantoic cells, whether or not neuraminidase was added. Consequently, estimates of multiplicity were based on the assumption that the whole 40 % of irreversibly bound virus was adsorbed by cells, thus providing an upper limit to the likely number of adsorbed infectious units per cell.

One-step growth experiments provide a simple means of investigating the characteristics of the release process: burst size, rate of release, and time of onset of release may be determined. Each of these properties could be influenced experimentally.

The rate of release and the size of the final yield depended on the nature of the medium used for the maintenance of the host cells. The best results were obtained with a complex medium containing purines, pyrimidines, amino acids and vitamins. Not only did release occur more rapidly in this medium than in a balanced salt solution, but with both viruses the final yield was about twice as high.

Individual cells infected with influenza virus apparently yield the bulk of their output over a short period of time; the onset of this release process can be synchronized by increasing the multiplicity of infection (Cairns, 1957). Consequently, the multiplicity of infection affects the release rate of influenza virus, without affecting its time of onset. The growth curves for NDV and Sendai viruses indicate a linear release process. This can be seen clearly in Fig. 9 and suggests that, once release begins, individual NDV and Sendai infected cells produce virus at a constant rate for many hours. Hence the release process for these viruses differs from that of influenza. Furthermore, increasing the multiplicity of infection does not affect the release rate of NDV or Sendai, although it does affect the time of onset of release. Consequently, NDV and Sendai resemble vesicular stomatitis virus (Cooper, 1958) and not inflenza virus, in that increasing the multiplicity results in shortening of the latent period.

Suitable doses of either actinomycin D or u.v. radiation also affect the length of the latent period. The ability of actinomycin D to shorten the latent period of Sendai virus multiplying in human amniotic cells was reported (Bukrinskaya & Zhdanov, 1963).

The possibility was considered that actinomycin D or u.v. radiation interfere with cellular nucleic acid synthesis, increasing the supply of precursors available for virus nucleic acid synthesis, so that virus development occurs more quickly in treated cells. The present experiments on the effects of different media were made to test this notion. However, variation in the supply of ingredients in the medium affected only the rate of release and not the time of onset. Attempts were also made to determine whether treatment with either actinomycin or u.v. radiation increased the intracellular nucleotide pool. The amount of trichloroacetic acid soluble nucleotides which could be extracted from both treated and control cells was determined spectrophotometrically (Newton, Dendy, Smith & Wildy, 1962). No differences could be detected. Consequently, it was not possible to provide support for the idea that availability of substrates is an important factor in determining the time of onset of virus release.

The possibility was also considered that inhibitors of RNA synthesis might have affected the susceptibility of cells to infection. Suitable doses of either inhibitor changed the response of cells to a particular dose of virus in such a way that they responded as though they had received a dose which was four times higher. That this was at least in part due to a change in susceptibility was demonstrated in infectivity titrations using stimulating doses of u.v. radiation. The conventional infectious unit for both viruses used in this study represents about ten physical particles (Isaacs & Donald, 1955). Under normal circumstances apparently only one particle in ten succeeds in infecting. Inhibitor treatment seemed to enable many otherwise undetected and non-functional virus particles to participate in multiplication. If this is so, not only may more particles be adsorbed than usually participate in the production of infection, but, given the opportunity, these particles may be capable

of functioning normally. It seems reasonable to suppose that cells actively block the establishment of infection, and that on average only about one virus particle in ten succeeds. If cellular DNA function is impaired, the chance of any adsorbed particle initiating infection increases. Virus invasion may stimulate an active, DNA-directed response on the part of the cell to resist infection. The observation of Heller (1963) that actinomycin prevented the production of interferon by infected cells is in keeping with these findings and suggestions: interferon may represent the means by which cells attempt to control nucleic acid synthesis.

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Relation of Biochemical Mutations to Actinomycin Synthesis in *Streptomyces antibioticus*

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SUMMARY

Biochemical mutants of *Streptomyces antibioticus* were isolated and tested for their capacity to produce actinomycin. The yield of actinomycin produced in minimal medium plus the required amino acid, by strains requiring an amino acid not present in the molecule of actinomycin, was not significantly different from that obtained from the wild-type strain. On the other hand, all the strains which required an amino acid, which was also a precursor of the antibiotic, showed on minimal medium a drastic decrease in the production of actinomycin. The results have been interpreted by assuming a different utilization by the cell of exogenous and endogenous amino acid pools for antibiotic and protein synthesis.

INTRODUCTION

Biochemical mutations can determine an alteration of antibiotic yield when introduced into strains of fungi or Streptomyces which produce antibiotics. Bonner (1947) isolated fifty-five mutants of *Penicillium notatum* which were unable to synthesize penicillin or which produced less than 5–10 units/ml. Sermonti (1957, 1959) and MacDonald, Hutchinson & Gillett (1963) found that a large proportion of auxotrophic mutants of *P. chrysogenum* showed a decrease of penicillin production. Similar results were reported by Alikhanian, Mindlin, Goldat & Vladimizov (1959) for the production of oxytetracycline by auxotrophic strains of *Streptomyces rimosus*; they also showed that antibiotic production by some auxotrophs was increased when the specific growth factor was added in excess of the amount required for normal growth. Mach, Reich & Tatum (1963), in a study of the biosynthesis of the polypeptide tyrocidine by *Bacillus brevis*, obtained several strains which were incapable of producing the antibiotic, but they did not succeed in isolating strains which were auxotrophic for amino acids present in the polypeptide. The present paper describes the effect of auxotrophy on the production of actinomycin by biochemical mutants of *S. antibioticus*. It will be shown that the nutritional dependence on the presence of an amino acid component of the polypeptide antibiotic had a more drastic effect on actinomycin yield than a nutritional dependence for other amino acids not components of the antibiotic. A possible interpretation of the results is given on the basis of a different utilization by the organisms of the exogenous and endogenous amino acid pools for protein and antibiotic synthesis.

METHODS

Culture media. Bennett's agar (Waksman, 1961) was used for the maintenance of strains and for plating ultraviolet-irradiated spores. Medium no. 40 of Magni & von Borstel (1962), a minimal medium adjusted to pH 7, was used in replica plating for the isolation of biochemical mutants. The complete medium V 6, used for vegetative growth, had the following composition (g./l.): yeast extract (A. Costantino, Favria, Turin, Italy), 5; beef extract (as above), 5; peptone (as above), 5; casein hydrolysate (as above), 3; glucose, 20; NaCl, 1·5; distilled water to 1000 ml.; adjusted to pH 6·6; sterilized at 115° for 30 min. The complete medium V 6 and the minimal medium of Goss & Katz (1960), in which galactose was replaced by glucose, were used for the fermentations.

Table 1. *Biochemical mutants of Streptomyces antibioticus*

Code	Genotype*	Derivation
AB 2	*(isol+val)⁻*	1692 by u.v. irradation
AB 8	*(isol+val)⁻*	1692 by u.v. irradation
AB 107	*(isol+val)⁻*	1692 by u.v. irradation
AB 111	*met⁻ thr⁻*	1692 by u.v. irradation
AB 1	*cys⁻*	1692 by u.v. irradation
AB 3	*arg⁻ ur⁻*	1692 by u.v. irradation
AB 9	*arg⁻ ur⁻*	1692 by u.v. irradation
AB 115	*arg⁻ ur⁻*	1692 by u.v. irradation
AB 114	*arg⁻*	1692 by u.v. irradation
AB 4	*his⁻*	1692 by u.v. irradation
AB 101	*his⁻*	1692 by u.v. irradation
AB 102	*his⁻*	1692 by u.v. irradation
AB 103	*his⁻*	1692 by u.v. irradation
AB 113	*ad⁻*	1692 by u.v. irradation
AB 6	*phe⁻*	1692 by u.v. irradation
AB 106	*leu⁻*	1692 by u.v. irradation
AB 7	*leu⁻*	1692 by u.v. irradation
AB 10	*leu⁻*	1692 by u.v. irradation
AB 110	*met⁻*	1692 by u.v. irradation
AB 109	*tyr⁻*	1692 by u.v. irradation
AB 2/1	*isol⁻/val⁻ met⁻*	AB 2 by u.v. irradation
AB 2/2	*isol⁻/val⁻ met⁻*	AB 2 by u.v. irradation
AB 2/5	*isol⁻/val⁻ ad⁻*	AB 2 by u.v. irradation
AB 2/3	*isol⁻/val⁻ met⁻*	AB 2 by u.v. irradation
AB 2/4	*isol⁻/val⁻ met⁻*	AB 2 by u.v. irradation
AB 2/6	*isol⁻/val⁻ met⁻*	AB 2 by u.v. irradation
AB 2/7	*isol⁻/val⁻ met⁻*	AB 2 by u.v. irradation
AB 2/21	*met⁻*	AB 2/2 spontaneous back mutation
AB 2/51	*ad⁻*	AB 2/5 spontaneous back mutation
AB 2/52	*ad⁻*	AB 2/5 spontaneous back mutation
AB 2/10	Prototroph	AB 2/2 spontaneous back mutation
AB 2/22	Prototroph	AB 2 spontaneous back mutation

* The following abbreviations have been used: isol = isoleucine; val = valine; met = methionine; thr = threonine; cys = cysteine; arg = arginine; ur = uracil; his = histidine; ad = adenine; phe = phenylalanine; leu = leucine; tyr = tyrosine.

Difco antibiotic medium 3 was used for the microbiological determination of actinomycin production.

Isolation of mutants. Biochemical mutations were induced by ultraviolet (u.v.)

irradiation. A spore suspension (0·5 ml. containing 5×10^7 spores in a Petri dish diam. 40 mm.) was irradiated with a low-pressure mercury lamp to a survival of 10^{-2}. Irradiated spores were plated on Bennett agar medium and incubated at 28°. After 4 days the colonies were replicated on minimal medium. Colonies which did not grow in minimal medium were isolated and their growth requirements characterized.

Production of actinomycin. Medium V6 (5 ml.) was added to an agar slope culture and the spore suspension used to inoculate 100 ml. of the same medium in a 500 ml. Erlenmeyer flask. The cultures were incubated at 28° on a reciprocating shaker for 24 hr. The mycelium was recovered by centrifugation, washed twice with the minimal fermentation medium and finally resuspended in 100 ml. of similar medium; 5 ml. of such a suspension was used as inoculum for each 100 ml. of fermentation media appropriately supplemented with the required nutrients. The fermentation flasks were incubated for 72 hr under the conditions given above for the growth of the inoculum. All experiments were done in triplicate.

Bioassay of actinomycin. Liquid cultures were centrifuged and the supernatant solution was used for antibiotic assay with *Bacillus subtilis* ATCC 6633 as a test organism. The antibiotic titre was determined by the filter paper disk method, with crystalline actinomycin D as standard.

Growth curves. Cultures in appropriately supplemented minimal medium of two auxotroph strains were prepared as described above and incubated for 48 hr on a reciprocating shaker. Samples (10 ml.) of the cultures were removed at various time intervals and were centrifuged in graduated tubes for 15 min. at 2500 rev./min. to determine the content of the mycelium, as % of the total volume of the liquid culture. Each experiment was done in triplicate.

Organism. All the isolates used were derived from *Streptomyces antibioticus* no. 1692 (culture collection of the Institute of Botany, University of Pavia). A list of isolated strains is given in Table 1.

RESULTS

The actinomycins are a family of chromopeptide antibiotics which differ only in the peptide portion of the molecule (Brockmann, 1960). *Streptomyces antibioticus* strain 1692 produces in a minimal medium actinomycins C_1, C_2 and C_3. The peptide portion of these actinomycins contains L-threonine, L-proline, sarcosine, L-*N*-methylvaline, D-valine and/or D-alloisoleucine (Fig. 1). Recent studies have shown that: L-proline is the precursor of the L-proline found in the actinomycins (Katz, Prockop & Undenfriend, 1962); L-valine is the precursor of both the D-valine and L-*N*-methylvaline residues of the actinomycin molecule (Katz & Weissbach, 1963); L-isoleucine is the precursor of the D-alloisoleucine (Albertini, Cassani & Ciferri, 1964); L-threonine is the precursor of the L-threonine and, to a lesser extent, of the D-alloisoleucine (Albertini *et al.* 1964); glycine or sarcosine are the precursors of sarcosine (Ciferri, Albertini & Cassani, 1964) in the molecule. Therefore, among the auxotrophs listed in Table 1, only those which require isoleucine and valine (*e.g.* AB2, AB8, AB107) and threonine (AB111) carry a deficiency for the synthesis of amino acids present in the proteins and in the antibiotic. On the other hand, the remaining strains are mutated in genes which control the synthesis of amino acids present only in the proteins.

All isolates listed in Table 1 were tested for actinomycin production in complete medium and in minimal medium plus supplement, i.e. amino acid(s) required by the strain, at 10 μg. and 100 μg./ml.

The results are collected in Tables 2–4.

In complete medium, the average production of actinomycin by auxotrophs which required amino acids not present in the polypeptide was of the order of

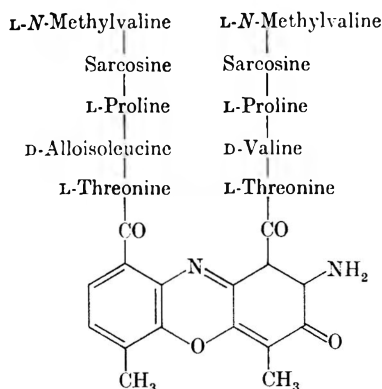

Fig. 1. Actinomycin C_2. In actinomycin C_1 the D-alloisoleucine is replaced by D-valine; in actinomycin C_3 the D-valine is replaced by D-alloisoleucine.

Table 2. *Antibiotic production by Streptomyces antibioticus auxotrophs requiring as nutrients nitrogen bases or amino acids not present in the peptide chain of actinomycin*

Strain	Antibiotic production (μg./ml.)			
Code	Requirement	Complete medium V6	Minimal medium +10 μg./ml. of required growth factor	Minimal medium +100 μg./ml. of required growth factor
AB 1	eys*	135	35	38
AB 3	arg+ur	180	55	58
AB 9	arg+ur	119	37	39
AB 115	arg+ur	120	35	33
AB 114	arg	110	31	33
AB 4	his	208	26	31
AB 101	his	122	28	29
AB 102	his	108	36	38
AB 103	his	98	27	28
AB 113	ad	118	35	30
AB 6	phe	52	6·5	8
AB 106	leu	88	18	20
AB 7	leu	74	16	17
AB 10	leu	92	19	22
AB 110	met	31	6·5	7
AB 109	tyr	80	15	18
	Average	108·4	26·6	28
	Wild type Average	106	25	–

* Abbreviations see Table 1.

108 μg./ml., not significantly different from 106 μg./ml., the average production of the parental prototroph strain. In complete medium, the mutants which required amino acids which are potential precursors of actinomycin showed a significant

Table 3. *Antibiotic production by Streptomyces antibioticus auxotrophs requiring amino acids which are present in protein and in the peptide chain of actinomycin*

Strain		Antibiotic production (μg./ml.)		
Code	Requirement	Complete medium V6	Minimal medium + 10 μg./ml. of required amino acid	Minimal medium + 100 μg./ml. of required amino acid
AB 2	isol + val*	83	0·9	2·7
AB 8	isol + val	70	0·8	2·1
AB 107	isol + val	79	1·0	3·8
AB 111	met + thr	68	0·7	3·2
AB 2/1	isol + val − met	88	1·0	3·3
AB 2/2	isol + val − met	12·6	1·0	1·4
AB 2/5	isol + val − ad	78	0·9	1·2
AB 2/3	isol + val − met	30	0·8	1·8
AB 2/4	isol + val − met	20	0·9	2·1
AB 2/6	isol + val − met	45	1·1	2·1
AB 2/7	isol + val + met	40	0·8	1·2
	Average	55·7	0·9	2·2
	Wild-type average	106	25	–

* Abbreviations see Table 1.

Table 4. *Antibiotic production by strains of Streptomyces antibioticus back-mutated to isoleucine and valine independence**

			Antibiotic production (μg./ml.)		
Code	Requirement	Derivation	Complete medium V6	Minimal medium + 10 μg./ml. of required amino acid	Minimal medium + 100 μg./ml. of required amino acid
AB 2/21	met	AB 2/2 *isol⁻/val⁻ met⁻*†	198	66	–
AB 2/51	ad	AB 2/5 *isol⁻/val⁻ ad⁻*	172	23	–
AB 2/52	ad	AB 2/5 *isol⁻/val⁻ ad⁻*	175	20·5	–
AB 2/22	–	AB 2/2 *isol⁻/val⁻ met⁻*	157·5	–	21·5
AB 2/10	–	AB 2 *isol⁻/val⁻*	100	–	12·5

* The strain AB 2/22 is back-mutated also to methionine independence.
† Abbreviations see Table 1.

but not very marked decrease in production of actinomycin (average 56 μg./ml.). In minimal medium plus supplement of the amino acid required for growth, however, all the isolates showed a decrease in the synthesis of actinomycin. The yield of actinomycin was decreased on an average to 25 % in the parental prototroph strain and in the auxotroph strains which required for growth amino acids not present in

the antibiotic. The actinomycin yield was not significantly increased in this group of amino acid requiring mutants when the required amino acid supplement was increased from 10 to 100 μg./ml. (Table 2).

On the other hand, all the isolates which required for growth an amino acid which was a precursor of actinomycin (Table 3) showed in minimal medium (supplemented with the amino acid required for growth) a marked decrease in the production of actinomycin (about 2 % of the yield in complete medium). The increase in the specifically required amino acid(s) from 10 to 100 μg./ml. resulted in a threefold increase of actinomycin yield; this was still very far from that of the other isolates listed in Table 2.

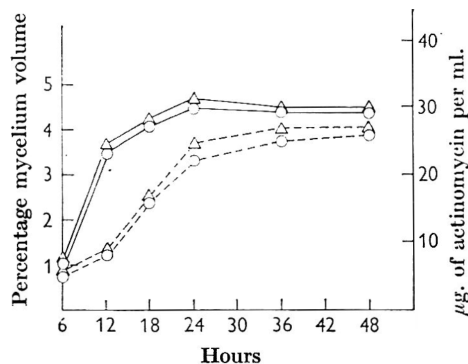

Fig. 2

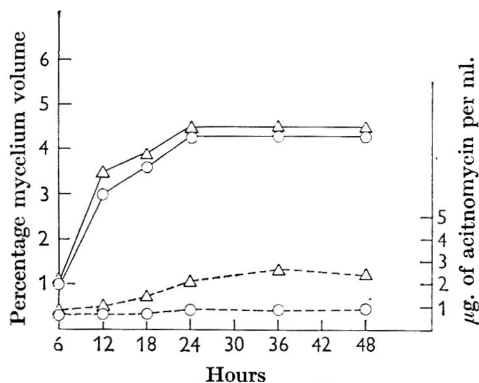

Fig. 3

Fig. 2. Growth curve and actinomycin production by the *Streptomyces antibioticus* mutant AB 3 arg⁻ ur⁻. △——△, ○——○, mycelium (% by volume of the total cultural liquid) in minimal medium supplemented with 100 and 10 μg./ml. of the required growth factors, respectively. △ - - - - △, ○ - - - - ○, antibiotic production in minimal medium supplemented with 100 and 10 μg./ml. of the required growth factors, respectively.

Fig. 3. Growth curve and actinomycin production by *Streptomyces antibioticus* mutant AB 2/2 isol⁻/val⁻ met⁻. △——△, ○——○, mycelium (% by volume of the total cultural liquid) in minimal medium supplied with 100 and 10 μg./ml. of the required growth factors, respectively. △ - - - - △, ○ - - - - ○, antibiotic production in minimal medium supplied with the required growth factors 100 and 10 μg./ml., respectively.

The fact that this behaviour was determined by the specific amino acid growth requirement is shown by the data of Table 4. Isolates carrying mutations for two kinds of amino acids, i.e. one required for actinomycin and protein synthesis and the other required for protein synthesis only, produced in minimal medium extremely low yields of antibiotic (Table 3). When these strains reverted to ability to synthesize the amino acid present in the antibiotic, the production of actinomycin became equal to that of the strains of the first group (Table 4).

A simple explanation for the above results could be that a very limited utilization of isoleucine, valine and threonine by all mutants requiring isoleucine, valine and threonine might result in scanty growth of the micro-organisms and therefore in a low production of antibiotic. This possibility was ruled out by the experiments reported in Figs. 2 and 3 which show that, despite very different yields of actinomycin in minimal media appropriately supplemented, two strains of different genotype showed equal growth rates which should indicate equal protein synthesis.

The decrease in yield of actinomycin by the auxotrophs which required for growth a specific amino acid also present in the polypeptide chain should therefore be ascribed to a different phenomenon.

In the case of *Bacillus anthracis*, Gladstone (1939) observed that omission from the growth media of isoleucine, valine or leucine inhibited the growth of the micro-organism. Such inhibition could be overcome by adding a mixture of all three amino acids. A similar 'toxic' effect brought about by threonine was removed by adding valine. It could be postulated, then, that such amino acids could exert a specific inhibition of the synthesis of actinomycin by *Streptomyces antibioticus*. However, the results obtained in the case of the prototroph *S. antibioticus* (Katz, 1960) showed that even very high levels of valine or isoleucine stimulate, rather than inhibit, growth and antibiotic production. Experiments performed on our mutant strains that carry a deficiency for valine and isoleucine revealed that the addition of a tenfold excess of leucine did not stimulate production of actinomycin. Nor did the addition of an excess of valine to fermentation media of the threonine-requiring strains have a beneficial effect.

DISCUSSION

MacDonald *et al.* (1963) suggested that 'the diminution of penicillin yield in *Penicillium chrysogenum* was most likely due to pleiotropic effects of the auxotrophic locus'. If this is an effect that occurs at random among mutants to auxotrophy, it is unlikely to explain our data because only mutations affecting the synthetic pathways of amino acids present in the polypeptide chain of actinomycin decrease the yield of actinomycin.

Our data are, at least in part, at variance with the data of Alikhanian *et al.* (1959), who observed in *Streptomyces rimosus* that the yield of antibiotic from some auxotrophs could be increased by the addition of an amino acid, for which the auxotroph was dependent, in amounts higher than those required only for growth. In our case, the production of actinomycin by the mutants of the first group (Table 2) seems to be unaffected by the concentration of the amino acid supplied to the culture.

The results obtained in minimal media may be explained as follows. The cell may recognize and utilize to different extents the amino acids absorbed from the environment and those synthesized by the cell itself, as reported, for instance, by Sercarz & Gorini (1964) for the control of repressor formation by endogenous *vs.* exogenous arginine. Such different pools, or compartments, may be utilized rather efficiently and to approximately the same extent for protein synthesis. On the other hand, for the synthesis of the antibiotic polypeptide chain, the utilization of the external pool may be very inefficient since the production of actinomycin is decreased to 1/50 of the normal at amino acid concentrations which support normal protein synthesis. Even a tenfold increase in the amino acid supplied stimulates only a threefold increase in the production of the antibiotic. Assuming that such a hypothesis is correct, one would then have to explain why in complete medium the production of antibiotic by the auxotrophs of Table 3 approaches a value near to that of the other mutants and of the prototroph. But it cannot be excluded *a priori* that the capacity of the cell to recognize two amino acid pools does not affect the utilization of small peptides or other possible precursors of the antibiotic present in the complete medium.

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A Co-precipitation Method for the Preparation of Transforming DNA from Small Samples of Low Density Bacterial Cultures

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SUMMARY

A method is described for the preparation of transforming DNA from 1 ml. samples of pneumococcal cultures of low density (e.g. about 10^7 bacteria). It seemed likely that the precipitation of very small amounts of transforming DNA from lysates of dilute pneumococcus cultures might result in loss of DNA unless a suitable co-precipitant was added. With high dilutions of transforming DNA, it was confirmed that such a loss was obtained. This loss was largely prevented by the addition of sodium hyaluronate in the presence of citrate. Dextran was not as efficient as hyaluronate as a co-precipitant.

INTRODUCTION

The study of certain reactions in bacterial transformation involves the use of lysates of relatively small numbers of bacteria as the source of the transforming DNA. It may sometimes be sufficient for a sample of the culture to be lysed with deoxycholate and used without further purification (e.g. Voll & Goodgall, 1961), but in some cases it may be desirable to remove substances present in the culture fluid before the lysates are assayed for transforming activity. When the lysates are prepared from 1 ml. samples of suspension containing perhaps not more than 10^7 bacteria, simple centrifugation before lysis might involve the risk of loss of some organisms. Hence, a precipitation method is indicated. Fox & Hotchkiss (1960) lysed the bacteria with deoxycholate followed by precipitation with 1·3 vol. of ethanol, centrifugation, and re-solution of the pellet. However, with such small numbers of bacteria the precipitation and recovery of DNA might be incomplete unless a carrier be added. Dr Harriet Ephrussi-Taylor (personal communication) has found that type-specific pneumococcal polysaccharide can act as a suitable co-precipitant with pneumococcal DNA, but its unavailability makes it unsuitable for general use. It was, therefore, decided to test sodium hyaluronate as a co-precipitant, and the results obtained are reported here.

METHODS

Organisms. Streptococcus pneumoniae C13, the receptor organism sensitive to streptomycin; *S. pneumoniae* r_2SQ, derived from C13 but resistant to streptomycin, optochin and erythromycin at the ery-r_2 level (Ravin & Iyer, 1961). Both strains

* Present address.

were maintained in medium as described by Martin & Ephrussi-Taylor (1964), with the addition of defibrinated rabbit blood to 2% (v/v).

DNA. Prepared from a lysate of a culture of *Streptococcus pneumoniae* strain r_2SQ made by incubating with 0·6% (w/v) sodium deoxycholate at 37° for 30 min, Crude DNA was precipitated by 2 vol. ethanol in the presence of citrate, most of the fibrous precipitate obtained being collected by winding on to a glass rod and the rest by centrifuging after decanting most of the mother liquor. A citrate + saline solution of the precipitate was deproteinized by repeated shaking with 0·33 vol. chloroform and 0·1 vol. capryl alcohol followed by final precipitation with 2 vol. ethanol. The DNA threads were wound immediately on to a glass rod and dissolved in sterile 0·15 M-NaCl. The concentration of the DNA solution was not determined, but from the experience obtained with previous preparations it was estimated that it was of the order 50–100 μg. DNA/ml.

Sodium hyaluronate solution. Sodium hyaluronate (Grade 1, Sigma Chemical Co., St Louis, Mo., U.S.A.) was dissolved in 0·15 M-NaCl to concentrations of 0·5 and 2·0 mg./ml.

Dextran solution. Dextran powder (Glaxo Laboratories Ltd., Greenford, Middlesex, England; Clinical/Technical Grade), having 46% of molecular weight between 51,000 and 150,000, was dissolved in 0·15 M-NaCl to 20 mg./ml.

Assay of transforming activity. A 'preculture' of competent receptor bacteria was obtained by inoculating 0·25 ml. of the stock *Streptococcus pneumoniae* strain Cl3 into 5 ml. 'P' medium (1% (w/v) Difco Neopeptone, 0·8% (w/v) charcoal-absorbed Difco yeast extract, 0·025% (w/v) glucose, pH 7·6), and incubating at 37° for $2\frac{1}{4}$ hr. From this a competent culture was obtained by inoculating 0·4 ml. preculture into 10 ml. 'NS' medium (1% (w/v) charcoal-absorbed Difco Neopeptone, 0·8% (w/v) charcoal-absorbed Difco yeast extract, 0·025% (w/v) glucose, containing $CaCl_2$ and a fresh extract of yeast as required), plus 0·1–0·2% (w/v) crystalline bovine albumin, and incubating at 37° until competence appeared. In some cases the preculture was frozen at −70° (in the presence of 10%, v/v, glycerol) and stored at this temperature in 0·5 ml. lots. When required, a 0·5 ml. sample was rapidly thawed and used for the preparation of the competence culture. Fifteen minutes before competence was expected, the culture was cooled in ice and 1 ml. samples added to 5 μl. or 10 μl. samples of DNA solution and the mixture incubated at 37° for 30 min. Suitable dilutions were plated in triplicate in a nutrient agar medium containing blood and incubated for 2 hr to allow expression of the gene conferring streptomycin resistance. At the end of this time, the plates were overlayered with nutrient agar containing streptomycin to give a final concentration in the plate of 200 μg. streptomycin/ml. The colonies grown on the plates after a further 24–48 hr incubation at 37° were counted, and the average of the triplicate platings calculated. Since there is no mutation to streptomycin resistance at this level, no control plates were necessary.

RESULTS

Effect of added hyaluronate on transforming activity. Amounts of sodium hyaluronate were mixed with suitable dilutions of the DNA solution to give the ratios indicated in Table 1. The mixture was then assayed for its transforming activity, which was compared with the activity of the DNA solution without added hyal-

uronate. No immediate effect on the transforming activity was noted, but a marked decrease occurred after the mixture with hyaluronate had been allowed to stand at 4° for 24 hr. Heating the hyaluronate in a boiling water-bath for 8 min. did not prevent the deterioration except with the lowest concentration of hyaluronate. However, the addition of 0·3M-trisodium citrate to final concentration 0·15M-trisodium citrate limited the decrease to 20–25% after 24 hr.

Table 1. *Effect of the presence of hyaluronate on the transforming activity of DNA*

Expt.	Amount of hyaluronate (mg.)	Ratio* of DNA:hyaluronate	Citrate added	No. transformants treated DNA/no. transformants untreated DNA: Immediate	After 24 hr	Percentage recovery of transforming activity: Immediate	After 24 hr
I	0·0025	1:0·25	−	600/504	.	119	.
	0·005	1:0·5	−	570/504	.	113	.
II	0·25	1:0·5	−	139/145	38†/122	96	31
	0·25	1:0·5	+	271/224	145/224	120	76
	0·25‡	1:0·5	−	.	117/112	.	96
	0·25‡	1:0·5	+	.	157/191	.	82
	0·375	1:0·75	−	106/145	31†/122	73	26
	0·375	1:0·75	+	265/224	128/191	118	67
	0·375‡	1:0·75	−	97/145	47†/122	67	40
	0·375‡	1:0·75	+	.	116/191	.	61
	0·5	1:1	−	74/145	24†/122	51	20
	0·5	1:1	+	240/224	167/191	107	88
	0·5‡	1:1	−	118/145	48†/122	81	39
	0·5‡	1:1	+	.	164/191	.	86

* The DNA concentration was not known exactly but the same solution was used for all experiments and was estimated to contain 1–2μg. DNA/ml. The ratio is expressed as 1 vol. DNA:mg. hyaluronate, where 1 vol. was estimated to be equivalent to 1–2μg. DNA. A change in the value of one side of the ratio shows that the concentration of the substance represented by that side of the ratio was changed while the concentration of the substance of the other side remained constant.

† Average number of transformants of six plates done at two dilutions.

‡ Hyaluronate solution heated for 8 min. in boiling water-bath and cooled before mixing with DNA.

Co-precipitation of DNA. A suitable dilution of DNA in 1 ml. samples was mixed with various amounts of hyaluronate with or without sodium citrate to 0·15M-citrate in conical centrifuge tubes. The mixtures were cooled in ice, 2 vol. ethanol added and shaken to ensure good mixing. The tubes were then allowed to stand in ice for 1 hr. to allow flocculation of precipitate, and centrifuged. The supernatant fluids were decanted, and the tubes allowed to stand inverted on an absorbent tissue for 1 hr. When possible excess liquid was wiped from the inside of the tubes with absorbent tissue, and 1 ml. 0·15M-NaCl or 1 ml. of a mixture of 1 vol. 0·15M-NaCl + 1 vol. 0·3M-citrate added. The precipitates were loosened from the walls of the tube by shaking, and left to dissolve at 4° for 24 and 48 hr. The DNA solutions were then assayed for their transforming activity and compared with a sample of the original dilution of DNA which had not been processed. The results obtained are shown in Table 2. It can be seen that without citrate there was a diminution of the transforming activity, whereas with citrate the recovery of even minute amounts of

Table 2. *Recovery of transforming activity after co-precipitation of DNA with hyaluronate by 2 vol. of ethanol*

Expt.	Amount of hyaluronate (mg.)	Ratio* of DNA:hyaluronate	Citrate added	No. transformants treated DNA / no. transformants untreated DNA at days after preparation of precipitate: 1 day	2 days	2 days frozen†	Percentage recovery of transforming activity at days after preparation of precipitate: 1 day	2 days	2 days frozen†
I	0·5	1:0·5	–	149/336	.	.	44	.	.
	0·5	1:0·5	–	158/336	.	.	46	.	.
II	2·0	1:2	–	302/385	185/365	.	79	51	.
III	1·0	1:1	+	63/84	238/268	200/268	75	89	75
	2·0	1:2	+	69/84	219/268	219/268	82	82	82
IV	2·0	0·1:2	+	.	152/206	.	.	74	.
	2·0	0·01:2	+	.	216/228	.	.	79	.
V	2·0	0·01:2	+	.	209/216	.	.	97	.

* As in Table 1. † DNA solution frozen between 1st and 2nd days.

Table 3. *Comparison of the recoveries of transforming activity after precipitation by ethanol, with and without added hyaluronate, and with added dextran, in presence of citrate*

Expt.	Amount of hyaluronate (mg.)	Amount of dextran (mg.)	Ratio* of DNA:co-pptant.	Vol. of ethanol	No. transformants treated DNA / no. transformants untreated DNA	Percentage recovery of transforming activity
I	0	0	—	1·4 ×	105/216	49
	2·0	0	0·01:2	2 ×	209/216	97
	0	20	0·01:20	2·8 ×	140/216	65

* As in Table 1.

DNA was about 85% of the original activity. This figure compares well with that obtained in the presence of hyaluronate but without the precipitation of the DNA. Therefore, no loss was obtained as a result of the precipitation. It can also be seen that there was no advantage obtained by keeping the dissolved precipitates frozen between the first and second day.

Comparison was made of the recovery of transforming activity after precipitation with (*a*) hyaluronate, (*b*) dextran as co-precipitant, (*c*) no co-precipitant. In all three cases citrate was added. The systems (*b*) and (*c*) had to be modified. The dextran had to be about ten times more concentrated than hyaluronate before a good precipitate was obtained, and more ethanol was required. Also, after centrifuging, a further period of cooling in ice was desirable before decantation, since the refractive index of the precipitate appeared to change and the precipitate could not be seen unless kept at 0°. It was also found that in the case of systems (*b*) and (*c*) and with this concentration of citrate, a small amount of an immiscible liquid was formed when the ethanol was added. The volume of ethanol added was critical. In absence of co-precipitant, the amount of ethanol had to be decreased to 1·4 vol., and with dextran, the maximum permissible was 2·8 vol. The results are given in Table 3. It can be seen that in the absence of co-precipitant there was only 50% recovery of activity; with the dextran system there was better recovery but much less than with hyaluronate.

Application to lysates. It was required to prepare DNA for the assay of transforming activity from samples of not more than 1 ml. of bacterial cultures of pneumococci having about 10^7 organisms/ml. On the basis of the above findings, the procedure adopted was as follows. To 1 ml. of the coccal suspension in a conical centrifuge tube was added 1·0 ml. 0·3 M-sodium citrate and 0·1 ml. 5% (w/v) sodium deoxycholate. The cocci were allowed to lyse for 30–40 min. at 37°. To the lysates were added 2·0 ml. sodium hyaluronate solution (containing 2·0 mg. sodium hyaluronate/ml.), followed by 8·0 ml. (i.e. 2 vol.) ethanol. After mixing, the tubes were allowed to stand in ice for 1 hr, centrifuged and the supernatant liquid decanted. The tubes were stood inverted at room temperature for 1 hr and excess fluid removed by careful wiping with absorbent tissue. The precipitate was dissolved in 1 ml. of a solution containing equal volumes of 0·15 M-NaCl and 0·3 M-citrate. To allow complete solution, the activity of the DNA was not usually assayed before 40 hr.

DISCUSSION

The results recorded in Table 3 confirmed that precipitation of very small amounts of DNA without a co-precipitant might lead to a substantial loss. In the case of a bacterial lysate the bacterial protein may act as a co-precipitant, but its efficiency would probably depend on the number of organisms present and thus vary. It is obviously desirable to provide a co-precipitant at a known concentration; sodium hyaluronate is adequate for this purpose. The deterioration of the transforming activity observed in the earlier experiments was presumably due to contamination of the hyaluronate used with DNase, since it was stopped by adding citrate. In some experiments (not reported here) with a crude preparation of RNA as co-precipitant, low activities were also obtained although the volume of precipitate was satisfactory. This system was not investigated further, but it seems

likely that the RNA used was also contaminated with DNase. Different samples of hyaluronate may contain different amounts of DNase, but the use of citrate will protect the DNA from this enzyme. It is, of course, necessary to have a DNase inhibitor present when the bacteria are lysed, to protect the DNA from the endogenous DNase.

Although it was not tested, there is no reason to suppose that this procedure would not be suitable for fewer bacteria but with very large numbers it may not be necessary to add the co-precipitant.

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Growth and Characterization of the Virus of Bovine Malignant Catarrhal Fever in East Africa

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SUMMARY

A strain of bovine malignant catarrhal fever virus (MCF) recovered from the blood of a blue wildebeest was developed by passage *in vitro* to a stage where it could be propagated serially in primary thyroid cell cultures by inoculation of cell-free fluids. Released virus titres ranged from $10^{3 \cdot 8}$ to $10^{5 \cdot 8}$ 50% tissue culture infectious doses/ml. This virus still caused fatal disease when inoculated to cattle, and was neutralized by antibody that appeared in the sera of cattle recovering from experimental infection. The principal cytopathic effects of the virus were the development of DNA-containing intranuclear inclusions and syncytia; the inclusions became increasingly basophilic as they matured. The cytopathic effects were inhibited in the presence of 5-iodo-2′-deoxyuridine (IUDR), and infectivity of the virus was abolished by treatment with ether or chloroform. Electron microscopy of inoculated cell cultures showed intranuclear, cytoplasmic and extracellular herpes-like virus particles. Suspensions of cell-free virus examined by negative-stain electron microscopy contained some particles of diameter 140 mμ–220 mμ, comprising an outer envelope and a central body or capsid; others consisted of only a naked capsid about 100 mμ in diameter. MCF virus is evidently a member of the herpes group, and has particular affinities to a subgroup which contains the agents of varicella, herpes zoster and the cytomegaloviruses.

INTRODUCTION

Investigations in Kenya have shown that a virus which causes malignant catarrhal fever (MCF) in cattle can be recovered from the blood leucocytes and from splenic tissue of apparently normal specimens of the blue wildebeest *Gorgon taurinus taurinus* Burchell (Plowright, Ferris & Scott, 1960; Plowright, 1963). The virus appears to be non-pathogenic for the natural host but produces typical MCF, with a mortality rate exceeding 90%, when inoculated intravenously or subcutaneously into cattle. Cytopathic changes were not detectable in cell cultures prepared from kidney, testis, bone marrow or blood of infected cattle but the virus was cultivated directly in bovine thyroid cell monolayers, causing a regular cytopathic effect with syncytia and intranuclear inclusions. MCF virus behaved at first in a manner similar to the human varicella-zoster agent (Weller, Witton & Bell, 1958; Taylor-Robinson, 1959) in that no cell-free infectivity was produced during early passages by thyroid cell cultures or by a line of calf kidney cells in

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which it was serially propagated. After several passages, from 7 to 30 with different isolates, calf kidney cells produced various amounts of free virus which passed through collodion membranes of average pore diameter 430 mμ (Plowright *et al.* 1960). However, it was then not possible to establish a continuous passage series in which cell-free fluid served as the inoculum. The present paper is about more recent experiments which led to the production of a line of MCF virus which was released regularly and at reasonably high titre into the fluid phase of primary calf thyroid cell cultures. This system has been utilized to study the cytopathic changes that accompany virus growth, and for observations about the stability, nucleic acid type and serological identity of MCF virus. Electron microscopy permitted morphological characterization of the virus particles, and revealed details of their intracellular growth.

METHODS

Virus isolation and passage. A strain (WC 11) of malignant catarrhal fever (MCF) virus was isolated in primary calf thyroid cultures inoculated with leucocytes from the blood of a wildebeest calf captured when less than 1 week old. Cells from a culture in which focal cytopathic changes had become evident were mixed with a suspension of calf kidney cells of the line BK165 and seeded into tubes where monolayers developed; cytopathic foci were visible after 3 days. Further passages in calf kidney cells were made at intervals of between 6 and 10 days, by detaching the cells with 0·02 % (w/v) EDTA and 0·01 % (w/v) trypsin and mixing with fresh cell suspensions in the proportion of between 1/3 and 1/10. We shall refer to this passage series as the cell-associated virus (CAV) line. A small amount of cell-free infective virus was first detected in the fluid phase of cultures at the 20th CAV passage, by inoculation of 5 ml. of the fluid into freshly established monolayers of calf kidney cells. It was again present in fluids from the 27th and 43rd passages, but remained at a low value; none was detectable in fluids from the 28th, 29th and 34th passages. Undiluted fluid from the 49th passage was clarified by centrifugation at 2000 g for 10 min. and then inoculated in 0·2 ml. quantities into tube cultures of primary calf thyroid cells; cytopathic foci were evident from the 3rd day after inoculation. From this point virus appeared regularly in the fluid phase of thyroid cell cultures following inoculation with clarified fluid from the previous passage. This series will be referred to as the released virus (RV) line; it has currently reached its 14th subculture. Fluids harvested on the 5th day, when the cytopathic effect was usually extensive, contained per ml. between $10^{3\cdot8}$ and $10^{5\cdot8}$ 50 % tissue culture infectious doses (TCID50/ml.) measured by titration in tube cultures of calf thyroid cells. The identity of this agent with earlier wildebeest isolates of MCF virus was indicated by its similar cytopathic effect *in vitro*, and confirmed by the production of fatal MCF in inoculated cattle after an incubation period of 9–11 weeks.

Virus neutralization tests. Serum was collected from three cattle before and after non-fatal infections with three different wildebeest-derived strains of MCF virus; post-infection samples were also obtained from another three animals which survived. All of these cattle resisted subsequent parenteral challenge of their immunity with virulent MCF virus.

A pooled sample of high-titre serum from cattle infected with infectious bovine rhinotracheitis (IBR) virus was tested for ability to neutralize MCF activity, as were also 12 sera from human (European) adults and three pairs of sera collected

during the acute and convalescent phases from African children with clinical varicella. All these sera were heated at 56° for 30 min. before use and immune cattle sera were diluted in tenfold steps in culture maintenance medium. Serum or serum dilutions were mixed with an equal volume of the RV line of virus, estimated to contribute $10^{2\cdot0}$ to $10^{2\cdot2}$ TCID 50/0·1 ml. The mixtures were shaken and left overnight at 4°, and 0·2 ml. quantities of each inoculated on the following day into two to five cultures of primary calf thyroid cells. The final examination for cytopathic effects was made on the 11th or 12th days after inoculation and the titre in quantitative tests was expressed as the reciprocal of the logarithm of the final serum dilution which protected 50 % of the tubes ($\log_{10}$ SN 50).

Tissue culture. Calf kidney cells of the established BK 165 line were propagated and maintained as recorded elsewhere (Ferris & Plowright, 1961). The calf thyroid cultures were prepared as described by Plowright & Ferris (1961) with a modified lactalbumen hydrolysate + yeast extract growth medium, supplemented with methionine, arginine, glutamine and vitamins as for the basal medium of Eagle (1955). This is similar to the medium designated CSV 6 by Cooper, Wilson & Burt (1959). For cell growth 10 % (v/v) normal ox serum was added; half this amount was adequate for maintenance. Coverslip monolayers of both cell types, intended for cytological study, were grown and infected with virus of the CAV or RV lines in Petri dishes, and incubated at 37° in an atmosphere of 5 % (v/v) carbon dioxide in air.

Metabolic inhibitors. 5-Fluoro-2′-deoxyuridine (FUDR) and 5-iodo-2′-deoxy-uridine (IUDR) were dissolved at concentrations from 10^{-7} M to 10^{-3} M, in Eagle's basal medium containing 5 % (v/v) normal ox serum. Tube cultures of calf thyroid cells were rinsed with phosphate buffered saline, and the inhibitor-containing fluids were each added to ten tubes, five of which were immediately inoculated with $10^{3\cdot8}$ TCID 50 of a strain of vaccinia virus (Plowright, Witcomb & Ferris, 1959) and five with $10^{3\cdot4}$ TCID 50 of the RV line of MCF virus. Infectivity titrations of the virus inocula were made simultaneously in calf thyroid cells. Microscopical examination of the tubes was made daily for 7 days; the inhibitor-containing media were renewed every second day.

Virus stability. Infective culture fluids containing virus of the RV line were subjected to treatment with 20 % (v/v) diethyl ether for 18 hr at 4°, following the technique of Andrewes & Horstman (1949), or 5 % (v/v) chloroform for 10 min. at 22° (Feldman & Wang, 1961). Treated and control samples were then titrated in calf thyroid cell cultures, with five tubes each tenfold dilution.

Cytology. Coverslip monolayers for light microscopy were harvested at daily intervals from 3 to 9 days after virus inoculation, and compared with uninoculated control preparations. Each coverslip was rinsed in 0·85 % (w/v) NaCl to remove excess medium, and transferred for 5 min. into Carnoy or Bouin fixative. Carnoy-fixed specimens were stained by the May–Grünwald–Giemsa technique (Jacobson & Webb, 1952); others, after Bouin or Carnoy fixation, were stained with haematoxylin + eosin. For cytochemical differentiation of nucleic acids (RNA, DNA) a parallel series of Carnoy-fixed monolayers was stained with acridine orange at pH 2·7 (Armstrong, 1956); after rinsing in phosphate-buffered saline they were mounted in liquid paraffin and examined with a Zeiss fluorescence microscope, with blue-violet excitation. Feulgen staining for DNA was also done after Carnoy fixation.

Electron microscopy. Tube cultures of calf thyroid cells were inoculated with cell-free fluid from the 5th passage of the RV line of MCF virus. Each tube received 0·2 ml. inoculum, containing about $10^{4\cdot0}$ TCID50 doses of infectious virus. Cytopathic foci were detectable in the unstained monolayers 3 days after inoculation; on the 5th day all tubes in which at least 75 % of the cell sheet seemed to be affected were harvested, together with uninoculated controls. After removal of culture fluid the cells were fixed *in situ* for a few minutes with a chilled solution of 1 % (w/v) OsO_4 buffered to pH 7·4 (Palade, 1952) and then carefully dislodged into the fixative by using a rubber pusher. The contents of several tubes were pooled, and fixation continued for about 1 hr. After a wash in Hanks's saline the cells were dehydrated with ethanol and embedded in a pre-polymerized 1:4 mixture of methyl- + *n*-butyl-methacrylate. Thin sections were collected on grids with carbon-stabilized 'Parlodion' supports. They were stained for 3 hr with a 5 % (w/v) solution of uranyl acetate in 1 % (v/v) acetic acid, or by the lead hydroxide method of Dalton & Zeigel (1960).

For negative-staining of intact virus a fluid harvest from the 10th passage of the RV line, with a titre of $10^{4\cdot0}$ TCID50/ml., was first clarified by centrifugation at 2000 *g* for 10 min. and then concentrated 50-fold by centrifugation at 30,000 rev./min. for 90 min. in the no. 30 rotor of a Spinco Model L centrifuge. The deposit was resuspended in phosphate-buffered saline containing 5 % (v/v) normal ox serum. A drop was mixed on a carbon-coated specimen grid with a drop of 1 % (w/v) potassium phosphotungstate (pH 6·8). Most of the fluid was blotted off with filter paper and the specimen was examined without delay. Micrographs were obtained at initial magnifications of up to × 12,000 for sections and × 25,000 for negatively-stained specimens, by the use of Philips EM 75b and EM200 electron microscopes.

RESULTS

Neutralization tests

Bovine sera collected before experimental infection of the animals did not inhibit cytopathogenicity of the released virus (RV) line of malignant catarrhal fever (MCF) virus; but all of the six sera from recovered cattle showed $\log_{10}$ SN50 titres ranging from 0·8 to 1·8. Infectious bovine rhinotracheitis (IBR) virus antiserum showed no neutralizing action against MCF virus, nor did any of the human sera tested.

Metabolic inhibitors

Neither 5-iodo-2′-deoxyuridine (IUDR) nor 5-fluoro-2′-deoxyuridine (FUDR) had any visible toxic effect on the uninoculated calf thyroid cells, even at maximum concentration. In the presence of IUDR at 10^{-7} M or 10^{-5} M the cytopathogenicity of vaccinia virus was not affected; but IUDR at a concentration of 10^{-4} M noticeably delayed the appearance of cytopathic effect, and at 10^{-3} M no cytopathic changes were evident during the 7 days of observation. Cytopathic effects due to MCF virus were delayed by IUDR at 10^{-6}M and 10^{-5}M, and completely suppressed by 10^{-4}M and above. This suggests that the MCF agent, like vaccinia, is a DNA-containing virus. In marked contrast, FUDR even at the highest concentrations tested did not obviously inhibit cytopathic changes caused by either vaccinia or MCF virus. Possibly FUDR was readily inactivated by calf thyroid cells, as

suggested by Hamparian, Hilleman & Ketler (1963) for primary monkey kidney cells. Alternatively, the explanation may be found in recent studies (e.g. Easterbrook & Davern, 1963; Stevens & Groman, 1964) which have shown that inhibition of virus growth by pyrimidine analogues, even when leading to complete suppression of the production of infectious particles, is not necessarily accompanied by suppression of the specific cytopathic effect.

Virus stability

Ether-treatment of fluid containing $10^{5 \cdot 2}$ TCID50 of MCF virus/ml. resulted in total loss of infectivity; chloroform-treatment of fluid containing $10^{4 \cdot 6}$ TCID50/ml. had the same effect.

Cytopathology

Calf kidney cell monolayers, fixed and stained at intervals after inoculation with the cell-associated virus (CAV) line of malignant catarrhal fever (MCF) virus, conformed exactly to the original account (Plowright *et al.* 1960) of focal degeneration in the cell sheet preceded by the development of intranuclear inclusions and large syncytia. In the series of thyroid cell monolayers inoculated with virus of the newly developed released virus (RV) line the gross cytopathic effect was similar, but extended more rapidly through the cell sheet; there was also more variation in the number and size of syncytia, which were sometimes comparatively inconspicuous. Even with the biggest inocula of RV line virus the cellular response remained obviously asynchronous; scattered foci of altered cells were present after 3 days, and the effect had spread to include perhaps 75% of the monolayer on the 5th day. After 8 days only a few cells, mostly abnormal, still adhered to the glass. As cells at various stages of infection always co-existed in the cultures, the interpretation in sequential terms must be speculative. In the following description reference to 'early' and 'late' manifestations is based on the degree of departure from normality shown by the cells concerned, and on recognition of a changing pattern of cell types over the period of observation. In Carnoy-fixed cultures stained by the May–Grünwald–Giemsa method the earliest unequivocal change after inoculation was the development of diffuse purplish staining material throughout the nucleus, obscuring the normal pattern of chromatin and nucleoli. These nuclei were mostly larger than normal, suggesting that nuclear swelling may be an early response to infection, and the nuclear membrane frequently showed irregular beading (Pl. 1, figs. 2, 3). After Bouin-fixation and staining with haematoxylin + eosin, the least-affected cells had nuclei which contained patches of eosinophilic granular material. With either method of fixation and staining, but especially after Bouin-fixation, more advanced nuclear changes were readily seen even at low magnifications: a characteristic 'halo' or retraction space became evident along the inner aspect of the nuclear membrane. The nuclear contents now constituted an inclusion (Pl. 1, fig. 2, 4, 5). Further enlargement of the retraction space was accompanied by further wrinkling and beading of the nuclear membrane, and a gradual shift in the staining reaction of the inclusion towards increased basophilia (Pl. 1, fig. 4). Nucleolar disintegration was also evident, some fragments becoming embedded in the inclusion while others adhered to the nuclear membrane. In some of the cells which showed nuclear alterations, small clusters of basophilic granules were seen

in juxtanuclear regions of the cytoplasm (Pl. 1, figs. 3, 5); these were not present in uninoculated control cells. In cultures which showed advanced cytopathic effects there were some cells in which the nucleus, besides the changes described, was surrounded by a fringe of radiating eosinophilic projections associated with basophilic granules (Pl. 1, fig. 6). With the increase of nuclear abnormality there was a tendency for the cells to round-up and become detached from the monolayer; fragmentation of the nucleus into basophilic debris and the presence of branching processes radiating from the cell periphery were signs of impending detachment.

An interesting feature of multinucleate cells in these cultures, whether small ones with only two or three nuclei or more extensive syncytial masses, was the coexistence of nuclei showing very different degrees of alteration. Presumably this is attributable to fusion (or recruitment) of neighbouring cells, of which some were normal and others in various stages of infection. Cytoplasmic vacuolation was prominent in some syncytia.

Staining for nucleic acids. With acridine orange, normal calf kidney and thyroid cells showed the usual flame-red fluorescence of cytoplasmic RNA-containing material. Nuclear chromatin gave the greenish yellow emission characteristic of DNA, and multiple red nucleoli were always in close relation to well-developed patches of nucleolus-associated chromatin. After inoculation, cells of otherwise normal appearance became conspicuous because of peculiarly diffuse DNA-containing material which accumulated in the nucleus (Pl. 1, fig. 7). In the later stages a strong fluorescence for DNA was given by the fully developed intranuclear inclusions (Pl. 1, fig. 8); small red-fluorescing globules occurred inside the inclusions and on the wrinkled nuclear membrane, and are assumed to represent nucleolar remnants. The peripheral 'halo' or retraction space was not fluorescent after acridine orange staining. Feulgen-stained monolayers likewise indicated the presence of a substantial amount of DNA in the intranuclear inclusions. With the acridine orange and the Feulgen methods where juxtanuclear cytoplasmic inclusions occurred they too gave positive reactions for DNA.

Electron microscopy

No unusual features were noted in sections of uninoculated control cell cultures. The cells were of normal appearance and contained no recognizable virus particles. In marked contrast, cells sectioned 5 days after inoculation with the RV line of MCF virus were morphologically abnormal and virus-like particles were identifiable in intimate relation to the cells.

Nuclear changes. Margination of chromatin and an absence of recognizable nucleoli were the most obvious signs of nuclear involvement. The interior of such nuclei was less dense than in normal cells, and closer inspection showed that normal nuclear fine structure had been replaced by a heterogeneous matrix with particulate and fibrillar components (Pl. 2, fig. 9). Rounded profiles, with a regularity of size and form that at once suggested virus particles, were scattered in seemingly random fashion throughout the nucleus (Pl. 2, fig. 10). Each virus-like particle was about 90 mμ in diameter and consisted of an internal dense body, or nucleoid, separated by a clear zone from a single dense peripheral ring. The nucleoid measured about 40 mμ; it was sometimes uniformly dense, but more often had a pale centre giving an annular profile on section. Aggregates of virus-like particles tended to occur

near to the nuclear envelope; orientation of the particles in linear array was noticed occasionally in the larger aggregates, suggesting the onset of viral crystallization *in situ* (Pl. 3, fig. 11). Interspersed with the intranuclear virus particles was a meshwork of coarse irregular filaments, the outlines of which often suggested an incomplete or precursor form of virus particle, but convincing evidence for such an interpretation is lacking.

Another interesting feature was present in a small proportion of the affected nuclei. This was a system of fine ramifying tubular formations, limited to the periphery of the intranuclear matrix. It was possible in places to observe continuity between the tubules and the inner layer of the nuclear envelope, from which they seemed to originate as multiple invaginations. Some were dilated, giving rise to intranuclear vesicles continuous with the space between the two layers of the nuclear envelope. Virus-like particles occurred around and within such vesicles, those on the outside were of the typical 90 mμ single-ringed variety; but those inside the vesicles were larger, each having a duplicated peripheral ring and a total diameter of about 130 mμ (Pl. 3, fig. 12). In effect, such particles can be regarded as extranuclear.

Small ring-like structures with a diameter of about 20 mμ occurred in profusion in certain cell nuclei (Pl. 3, fig. 13), typically in areas devoid of the well-defined 90 mμ virus particles. They had no morphological resemblance to previously recognized viruses; since they were generally most numerous in cells at an advanced stage of infection it is possible they represent some by-product of intranuclear virus replication.

Cytoplasmic changes. The response of the cytoplasm in thyroid cells inoculated with MCF virus was invariably less dramatic than that of the corresponding nuclei. However, a characteristic feature, never seen in uninoculated control cells, was aggregation of the mitochondria; it occurred chiefly around the nucleus and near the Golgi complex (Pl. 4, fig. 15).

Virus-like particles, clearly comparable with those present in the nucleus, were demonstrable in the cytoplasm in relatively small numbers, but there was no obvious tendency for them to accumulate in the cytoplasm. Those seen most commonly were of the larger double-ringed type, measuring 130 mμ in diameter; they were situated, singly or in small groups, in closed cytoplasmic vesicles (Pl. 5, figs. 16, 17). They did not occur free in the cytoplasm, nor within the confines of the endoplasmic reticulum. The virus-containing vesicles were widely distributed between the nucleus and the cell surface.

Apart from the intravesicular particles, a small proportion of cells also contained a prominent cytoplasmic body consisting of numerous virus particles embedded in a dense granular matrix. The body was juxtanuclear in position, closely related to the Golgi complex and to the mitochondrial aggregates already mentioned (Pl. 4, fig. 15). On section, each of these bodies measured up to several microns across but was irregular in shape; no membrane which separated it from surrounding cytoplasm was discernible. The contained virus particles were mainly of the single-ringed 90 mμ type, but larger ones with double-rings were occasionally present. For the most part, it was not feasible to differentiate the virus particles from the granular material in which they were embedded, since many of them seemed to be morphologically incomplete. Often a central nucleoid was entirely lacking and in other cases it was represented only by a short dense filament reaching out of the particle itself

into the surrounding matrix or adjacent cytoplasm. In short, the dense bodies were composed mainly of fragmentary virus particles; their frequency and location suggested probable identity with the juxtanuclear basophilic masses identified by light microscopy.

Extracellular virus. Numerous virus particles occurred on the surface of cells which showed the nuclear and cytoplasmic changes described. Individual particles were usually adherent to the outer aspect of folded portions of the cell membrane, while large clusters of apparently free virus particles were located in intercellular clefts throughout the cell sheet (Pl. 3, fig. 14). All extracellular particles were of the double-ringed type, with a diameter about 130 mμ.

Negative-staining electron microscopy. These preparations contained a substantial number of particles with undoubted viral characteristics. They were of two distinct morphological types, enveloped and naked; the two types occurred in clusters with about equal frequency but were rarely intermingled. Enveloped particles (Pl. 5, fig. 18) possessed a loose external membrane of irregular outline, enclosing a central body (or capsid) having a well-defined and regular profile. Overall size of enveloped particles ranged from 140 to 220 mμ, while the central body measured about 100 mμ. Naked particles (Pl. 5, fig. 19) appeared circular or, quite often, hexagonal in outline; they measured 100 mμ across and closely resembled the central bodies of enveloped particles. At high magnification they were seen to be coated with regular subunits (capsomeres) of a hollow and elongated character. Each subunit had a diameter of about 9·5 mμ, and the length of those viewed in profile round the particle periphery was about 12·5 mμ. Similar but less well revealed subunits were discernible on the central body of many of the enveloped particles.

DISCUSSION

The strain of virus used in this study is typical of those that have been isolated from wildebeest or cattle naturally infected with malignant catarrhal fever (MCF) in East Africa. The released virus (RV) line of this isolate was still capable of producing fatal disease on parenteral inoculation into cattle and was neutralized by antibody which appeared in the serum of other cattle which had recovered from experimental MCF. It was not neutralized by antisera against infectious bovine rhinotracheitis virus or by adult human sera, some of which presumably contained antibodies to herpes simplex, varicella and herpes zoster viruses. The cytopathic effect of the RV line of MCF virus was essentially similar to that produced by primary MCF isolates in calf thyroid cells, and the virus-like particles now demonstrated in intimate relation with the altered cells, and in culture fluids, represent without doubt the causal agent of malignant catarrhal fever in East Africa.

The original report on wildebeest isolates of malignant catarrhal fever (MCF) virus (Plowright *et al.* 1960) called attention to some herpes-like properties, notably in regard to their epizoötiological behaviour and cytopathic effects in monolayer cultures. Subsequent experience with this virus in the laboratory has confirmed the earlier impressions: in addition to the cytopathological details, affinity with the herpes group is indicated by the demonstration of ether and chloroform sensitivity, and by the suppression of cytopathic changes in the presence of 5-iodo-2′-deoxy-

uridine which selectively inhibits DNA synthesis. Owing to the exclusive production of cell-associated virus during early culture passages, the original MCF virus isolates were likened to the subgroup of herpes viruses which includes the varicella and zoster agents. It has recently been suggested (Melnick *et al.* 1964) that cytomegaloviruses should also be included in this category, although their ability to produce cell-free infectious virus is greater than with the other agents mentioned (Smith, 1959). Varicella produces much cell-free infectivity in the tissues of man (Weller *et al.* 1958; Taylor-Robinson, 1959) but none was demonstrable in tissue extracts or sonically-disrupted cells from MCF virus-infected cattle (Plowright, 1963). Caunt (1963) found that a considerable quantity of stable infectious virus may be liberated ultrasonically from the cells of human thyroid cultures infected with varicella virus. The development of the RV line of MCF virus is a comparable achievement showing that failure to produce appreciable amounts of free infectivity *in vitro* is not an absolute and immutable characteristic of this subgroup. A morphological basis for the absence of stable free infectious virus has not been established for any of the agents discussed, but it might well be dependent on a failure to produce or release particles having an outer envelope; the latter, it has been suggested, may be required for infectivity in the case of herpes simplex (Smith, 1964).

The finding of basophilic Feulgen-positive granules in the cytoplasm of cells infected with MCF virus recalls similar observations on cells infected with cytomegaloviruses, either *in vivo* or *in vitro* (Smith, 1964; McAllister, Straw, Filber & Goodheart, 1963). Similarly, the increasing basophilia of the intranuclear inclusions in MCF virus-infected cells may indicate a closer resemblance to the cytomegaloviruses than to the classical herpes viruses, which produce mature inclusions often regarded as necessarily eosinophilic (Andrewes *et al.* 1961; Pereira, 1962). Nevertheless, in certain circumstances esosinophilic type A inclusions do not always develop in HeLa cells infected with herpes simplex virus, probably because the nuclei contain an excess of DNA which persists into the late stages of infection (Newton & Stoker, 1958). Felgenhauer & Stammler (1962) also failed to demonstrate Feulgen-negative inclusions in mouse fibroblast cultures infected with the same virus and fixed briefly in Carnoy's fluid. In addition, 'amphophilic' staining properties are on record for the nuclear inclusions of infectious bovine rhinotracheitis virus, an established bovine member of the herpes group (Cheatham & Crandell, 1957). It is perhaps appropriate to question whether tinctorial conformity with the original concept of type A nuclear inclusions (Cowdry, 1934) should continue to be regarded as significant for purposes of virus classification; cytological, biophysical and chemical criteria of a more basic and reproducible nature are now available to the taxonomist (Andrewes *et al.* 1963).

In respect of size, morphology, and relationship to the host cell the malignant catarrhal fever particles bear a strong resemblance to elementary particles of the herpes group. Details of herpes virus morphology and development have been described, amongst others, for herpes simplex virus (Morgan, Rose, Holden & Jones, 1959), simian B virus (Reissig & Melnick, 1955), avian infectious laryngotracheitis virus (Watrach *et al.* 1959), infectious bovine rhinotracheitis virus (Armstrong, Pereira & Andrewes, 1961) and equine abortion virus (Arhelger, Darlington & Randall, 1963). In common with these reports, electron microscopy of MCF virus-inoculated cells points to the nucleus as the primary site of viral replication;

single-ringed particles measuring 90 mμ seem to be assembled there in a manner indistinguishable from that of acknowledged herpes viruses. A second ring is acquired by each particle on leaving the nucleus, and evidently consists of a membranous investment from the inner layer of the bilaminar nuclear envelope. The factors which initiate and control the extrusion of particles from the nucleus remain obscure. The occasional presence of vesicular ingrowths from the nuclear envelope, containing double-ringed virus particles, was noted earlier for herpes simplex virus (Falke, Siegert & Vogell, 1959) and for a human cytomegalovirus (Stern & Friedmann, 1960).

As there were no recognizable cytoplasmic foci of MCF virus replication, it is likely that all of the double-ringed particles observed in cytoplasmic vesicles were being transported at the moment of fixation, either from the nucleus towards the cell surface or in the reverse direction. Recent work with herpes simplex virus, in which electron-opaque tracers were mixed with the virus inoculum, showed clearly that in cell cultures the virus did indeed enter cells as a consequence of phagocytosis and became incorporated into cytoplasmic vesicles (Holmes & Watson, 1963; Epstein, Hummeler & Berkaloff, 1964). The subsequent fate of ingested particles, in the 'eclipse phase', preceding appearance of newly synthesized virus particles, has not yet been clearly defined in morphological terms; but it is commonly supposed that enzymic digestion of the engulfed particles explains their disappearance and accounts for the uncoating of infectious viral nucleic acid. In the present experiments it was not possible to distinguish between 'ingoing' and 'outgoing' cytoplasmic MCF virus particles, but there is no reason to doubt that both forms were present in many of the cells examined.

It is tentatively proposed that the dense juxtanuclear mass of fragmentary MCF virus particles, observed in a proportion of the cells, may have a bearing upon the fate of phagocytosed virus. It is only to be expected, in the later stages of infection that some cells in the cultures would be ingesting unusually large amounts of virus released into the medium from nearby infected cells; it is now suggested that this was followed by localization of a mass of partially digested virus material in the vicinity of the Golgi complex, where there were signs of viral nucleoids being released into the surrounding cytoplasm. Similar viral aggregates seem so far not to have been noticed in studies on the typical herpes viruses; but closely comparable dense bodies were met with in a recent investigation of the liver in mice, following intraperitoneal inoculation with a murine cytomegalovirus (Ruebner *et al.* 1964). These workers considered the dense masses to be sites of viral destruction, possibly in the lysosomal category; the hypothesis advanced here for MCF virus accords well with their interpretation.

In correlating information on virus particles based on thin sections with that obtained by negative-staining electron microscopy, some allowance for technical distortion is necessary. Shrinkage tends to occur during dehydration and embedding before cutting sections, while collapse and spreading of unsupported membranes and other non-rigid components can be expected in specimens allowed to dry in phosphotungstate. In the circumstances, there was reasonable correspondence in size and structural detail between the single- and double-ringed particles seen in thin sections, and the naked and enveloped forms revealed by negative staining; they may be regarded as identical. The measured size of the larger enveloped particles

coincides with that obtainable by applying the factor of Black (1958) to our most recent data on the passage of MCF virus through graded collodion membranes: all infectivity was held up by membranes of 275 mμ average pore diameter whilst others of 300 mμ allowed virus to pass. From this it is calculated that the minimum diameter of the infectious MCF particles was approximately 180 mμ. The exact number of hollow elongated subunits comprising the capsid of MCF virus has not yet been determined. Nevertheless, the present observations leave little doubt that the viral architecture will prove to be indistinguishable from the icosahedral pattern, with 162 subunits, that has been established for herpes simplex (Wildy, Russell & Horne, 1960), cytomegalovirus (Smith & Rasmussen, 1963), varicella virus (Almeida, Howatson & Williams, 1962) and the virus of avian infectious laryngotracheitis (Watrach, Hanson & Watrach, 1963).

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EXPLANATION OF PLATES

PLATE 1

Figs. 1–6 are photomicrographs of 9-day monolayers of primary calf thyroid cells; all except fig. 4 were Carnoy-fixed and stained by the May–Grünwald–Giemsa method.

Fig. 1. Uninoculated culture (×1000).

Fig. 2. Culture infected 4 days previously with the released virus (RV) line of malignant catarrhal fever (MCF) virus, showing nuclear enlargement and masking of the normal chromatin pattern; two nuclei contain early inclusions surrounded by a narrow halo and show beading of the nuclear membrane (×1000).

Fig. 3. Cells in a 4-day-infected culture, in which diffuse intranuclear staining largely masks the chromatin network: note also slight beading of the nuclear membrane, early halo-formation around the nuclear contents, and the presence of basophilic granules in the juxtanuclear cytoplasm (×1000).

Fig. 4. Well-formed basophilic intranuclear inclusions, each surrounded by a wide clear zone or halo. Haematoxylin-eosin, after Bouin fixation (×1250).

Fig. 5. Infected cell with an intranuclear inclusion, showing also a prominent juxtanuclear cluster of basophilic granules (×1500).

Fig. 6. A cell with diffuse intranuclear staining and beading of the nuclear membrane, also showing a perinuclear fringe of eosinophilic processes accompanied by small basophilic granules (×1500).

Figs. 7 and 8 are fluorescence photomicrographs of BK165 cell cultures, infected with the cell-associated virus (CAV) line of MCF virus and stained with acridine orange. Fig. 7 shows a syncytium with seven nuclei, two of which (right of centre) are of normal appearance. Others (arrows) are filled with diffuse material giving the yellow-green fluorescence characteristic of DNA. The syncytium in fig. 8 contains two nuclei (arrow) with well-developed inclusions emitting intense yellow-green fluorescence. Prints made from original colour transparencies (×625).

PLATE 2

Electron micrographs of calf thyroid cells.

Fig. 9. Section illustrating the abnormal nuclear morphology found in many cells 5 days after inoculation with malignant catarrhal fever virus. Notice margination of the chromatin, and a heterogeneous intranuclear matrix (×32,000).

Fig. 10. Nuclear fine structure observed at higher magnification, and showing a collection of 90 mμ virus-like particles: the particles consist of an outer dense ring and a central nucleoid (×50,000).

PLATE 3

Electron micrographs of calf thyroid cells.

Fig. 11. Relatively large aggregate of typical intranuclear virus-like particles: linear orientation of particles on the right in this field suggests incipient virus crystallization (×39,000).

Fig. 12. Virus particles of the double-ringed type (approximate diameter 130 mμ) lying in an intranuclear vesicle the limiting membrane of which is indicated by arrows (×80,000).

Fig. 13. Intranuclear detail in a cell that showed obvious signs of degeneration 5 days after inoculation. Small (20 mμ) ring-like bodies of unknown significance are numerous; for comparison, a typical single-ringed virus particle is seen at the top right (× 32,000).

Fig. 14. Clusters of extracellular virus particles, situated in spaces between neighbouring cells of the monolayer (× 32,000).

PLATE 4

Fig. 15. Section of thyroid cell in an inoculated culture, showing a prominent juxtanuclear dense mass in the cytoplasm; it appears to be composed mainly of fragmentary virus particles. Note the occurrence of nearby mitochondrial aggregates (M). N = Nucleus. Electron micrograph (× 40,000).

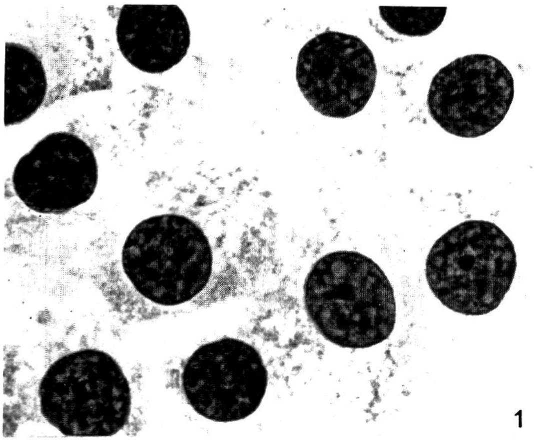
PLATE 5

Electron micrographs

Fig. 16. Section through neighbouring cells in an inoculated culture showing typical, 130 mμ double-ringed virus particles enclosed in cytoplasmic vesicles (arrows). That on the left appears to be fragmenting, and may be an 'ingoing' particle within a phagocytic vesicle derived from the cell membrane. Two particles at the upper right are intact, and closely invested by the vesicle membrane; these may represent 'outgoing' virus particles (× 50,000).

Fig. 17. Intravesicular cytoplasmic virus particle at high magnification. In the case of 'outgoing' particles, i.e. proceeding from their origin in the nucleus towards the cell surface, the enclosing vesicle is presumed to originate as an evagination from the outer layer of the nuclear envelope (× 100,000).

Figs. 18, 19. Phosphotungstate negative-stain preparations of tissue culture fluid containing cell-free malignant catarrhal fever virus. A typical enveloped particle is seen in fig. 18 and a naked one in fig. 19. On the latter can be seen hollow surface subunits (capsomeres) the elongated nature of which is revealed by those viewed in profile at the edge of the particle (× 200,000).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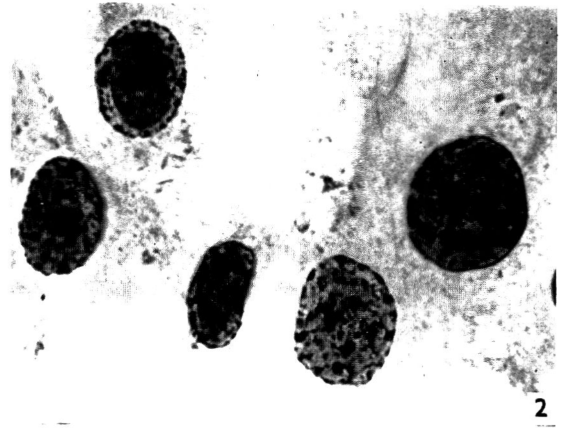
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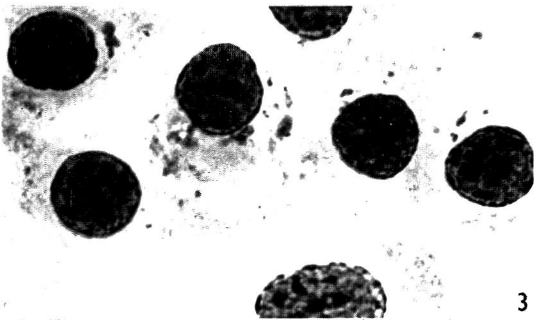
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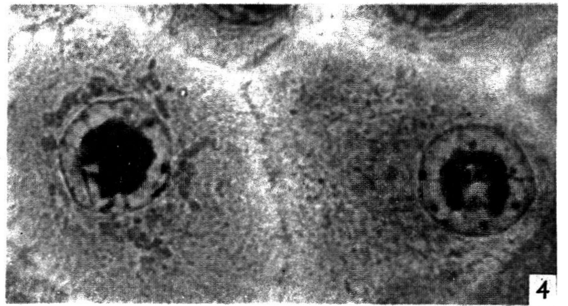
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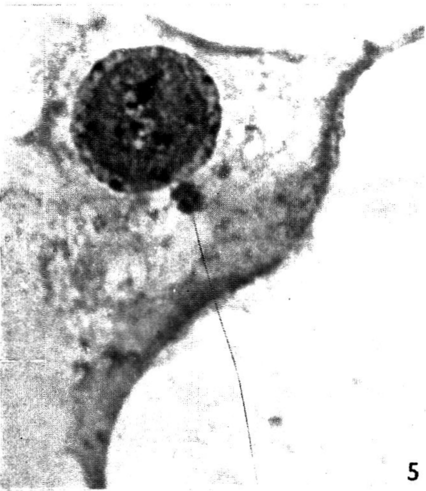
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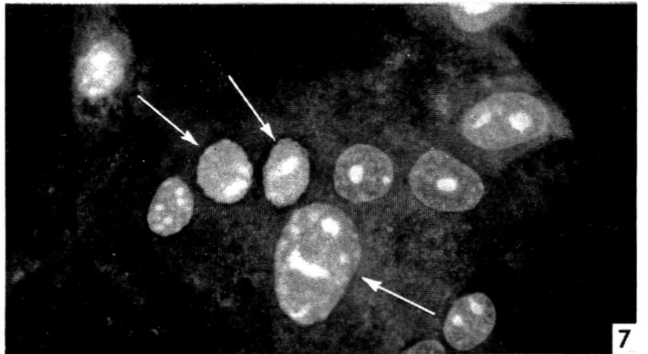
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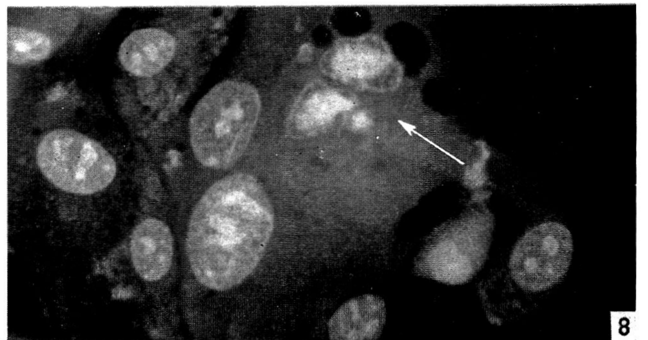
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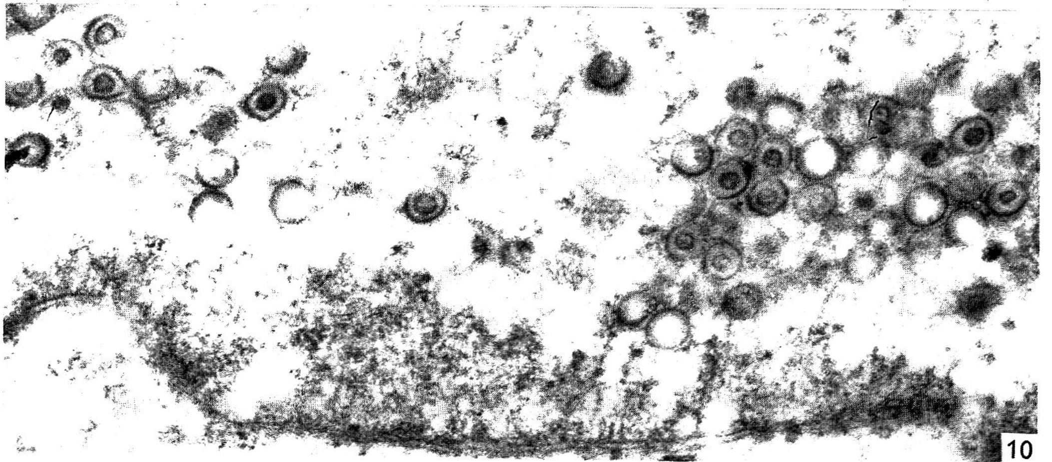

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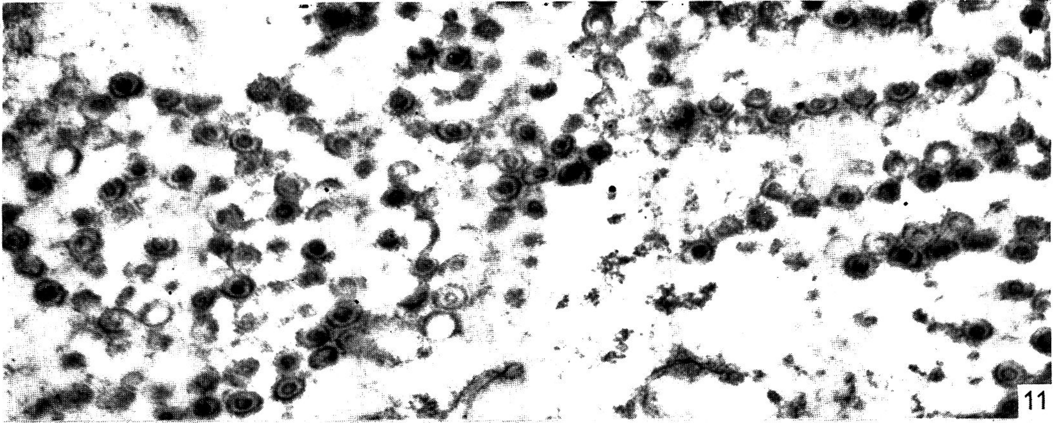

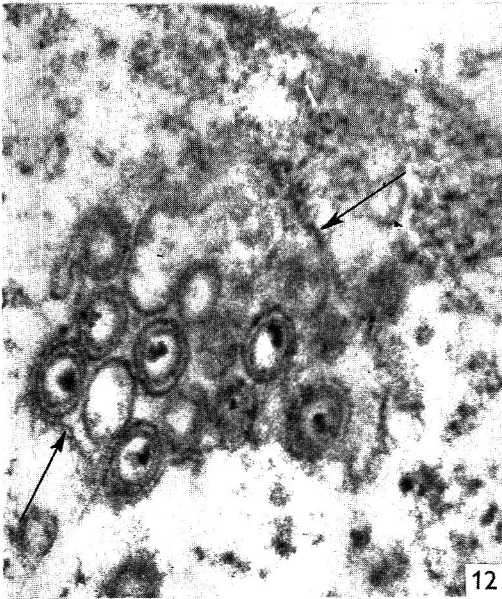

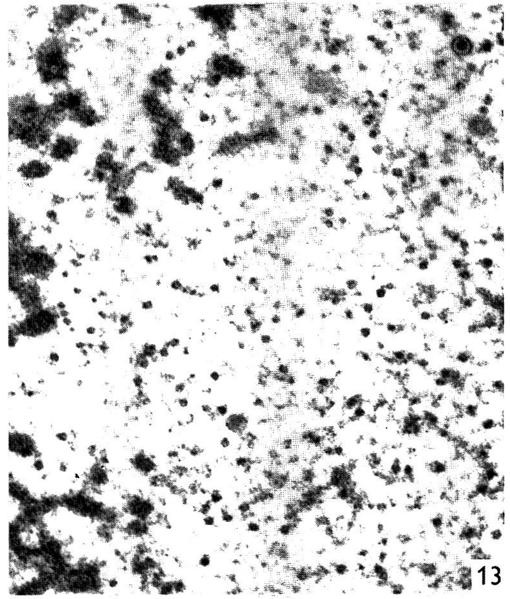

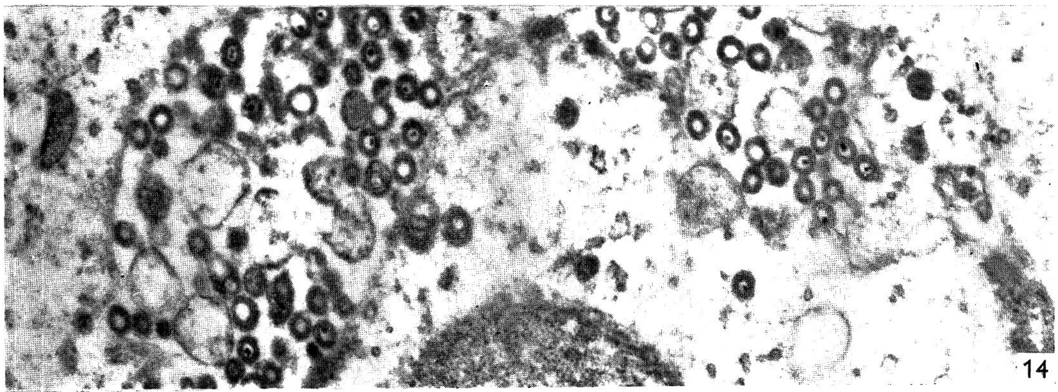

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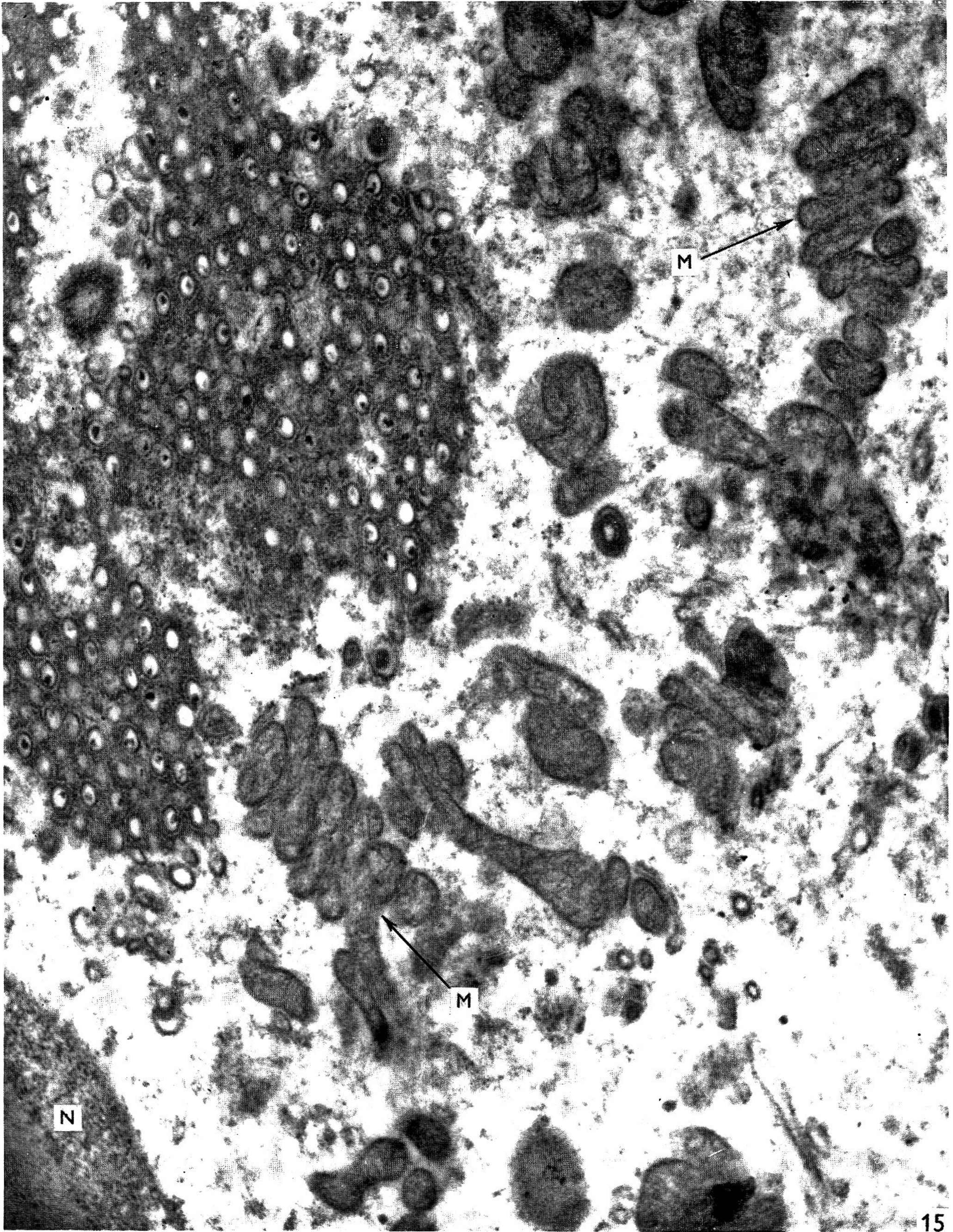

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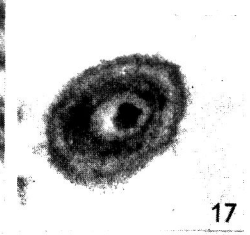

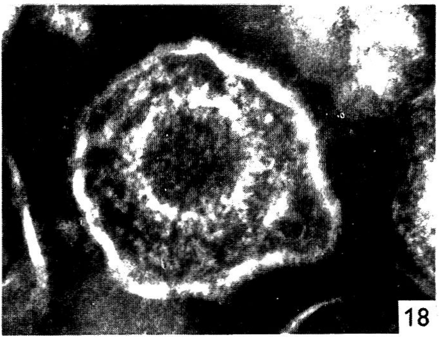

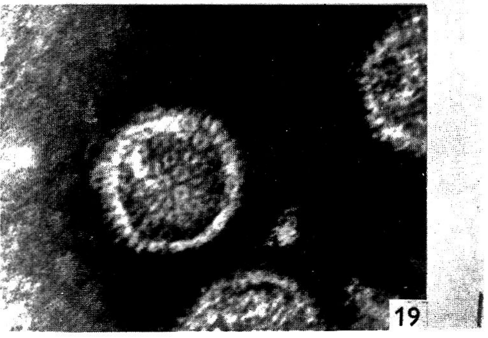

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Comparative Counts of Bacterial Nuclei Made Visible by Four Different Techniques

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SUMMARY

Four different methods were used to make visible the nuclei of *Escherichia coli* strains B and WP 2, and *Bacillus subtilis*. They were: (1) Giemsa staining after acid hydrolysis; (2) fluorescence microscopy after acridine orange treatment; (3) acriflavine staining and phase-contrast microscopy; (4) phase-contrast observations with high refractive index mounting medium (polyvinylpyrrolidone). Statistical analyses showed small but significant differences in nuclear counts between the methods. Methods 1 and 2 were somewhat preferable to methods 3 and 4 in that 1 and 2 were easier to score and there was less difference between observers. It was calculated that a single mean nuclear count, by any observer or method, based on 200 scored bacteria should 19 times out of 20 estimate the 'true' mean nuclear number of *E. coli* WP 2 to within about $\pm 7{\cdot}5\%$ and of *B. subtilis* and *E. coli* B to within about $\pm 12\%$. By increasing the number of bacteria counted and the number of methods used these limits could be appreciably decreased.

INTRODUCTION

In recent years a number of experiments designed to follow the fate of heritable changes in the bacterial genome during their subsequent segregation have been reported, for example after mutation induction (Ryan, Fried & Schwartz, 1954; Munson & Bridges, 1964) and also after recombination (Tomizawa, 1960). To interpret the observed patterns of segregation it was necessary to know the average number of nuclei/bacterium in the initial population and, if possible, the proportions of bacteria containing 1, 2, 3, 4,... nuclei. To do this most workers have used modifications of the Feulgen staining technique, in which fixed bacteria are exposed to a mild acid hydrolysis which results in the liberation of aldehyde groups on the DNA. These are made to undergo a Schiff reaction and the DNA becomes stained a very pale pink. As it is known that the RNA does not stain after such hydrolysis (Vendrely & Lipardy, 1946), other less specific stains have been used, Giemsa being the most favoured because of the greater intensity of staining (Piekarski, 1937, 1940; Robinow, 1942, 1944). The staining reaction with Giemsa after hydrolysis is abolished when the bacteria are treated with DNA-ase (Munson & Maclean, 1961). These staining methods are not noted for their ease and simplicity, success being critically dependent upon the durations of the fixation and hydrolysis stages. Other methods of making nuclei visible have therefore been sought.

The property of nucleic acids of fluorescing under ultraviolet or violet radiation

when treated with certain acridine dyes was utilized by Krieg (1954) and by Anderson, Armstrong & Niven (1959). They found that under certain conditions the DNA (nuclei) of *Escherichia coli* could be made to fluoresce green and the cytoplasm red.

The Feulgen-positive areas in fixed bacteria were shown by Tulasne (1949), Stempen (1950) and Mason & Powelson (1956) to coincide with areas of low refractive index within the cytoplasm of unfixed bacteria. These are normally only faintly visible with phase-contract microscopy but Barer, Ross & Tkaczyk (1953) were able to increase the contrast by altering the refractive index of the medium in which the bacteria were mounted. Mason & Powelson (1956) successfully used gelatin, and Schaechter, Williamson, Hood & Koch (1962) polyvinylpyrrolidone (hereafter PVP) as highly refractive mounting media with living cells. We have found that the contrast in water-mounted unfixed preparations may also be enhanced by lightly staining with acriflavine or acridine orange. This is presumably due to changes in refractive index within the cell, possibly by differential binding of the dye to nuclear and cytoplasmic material.

In the present work we have compared statistically the mean numbers of nuclei/bacterium in populations of bacteria, as found by using four different techniques representative of the known microscopic methods of making nuclei visible.

METHODS

Preparation of smears. The starting material for all the techniques used was a smear obtained by allowing organisms suspended in their growth medium to dry on a glass slide in a 37° incubator. These dried smears were stable for many hours. This enabled preparations from the same specimen to be observed by different techniques throughout the working day. The organisms used were *Escherichia coli* strains B and B/r WP 2 growing exponentially at 37° in glucose + salts medium (in the case of strain WP 2 with a tryptophan supplement 6 μg./ml.), and *Bacillus subtilis* growing exponentially in Oxoid nutrient broth at 37°.

Giemsa-staining technique. The smear was fixed for 3 min. in osmium tetroxide vapour followed by 5 min. in Schaudinn's alcohol at 37° and then stored in 70% (v/v) ethanol in water. It was then hydrolysed, for 5 min. in the case of *Escherichia coli* strains or for 23 min. in the case of *Bacillus subtilis*, in N-hydrochloric acid at 57–58°, washed in buffered water (0·0908 g./l. KH_2PO_4, 0·1188 g./l. $Na_2HPO_4.2H_2O$, pH 6·98) and stained with 10% aqueous Giemsa solution (G. T. Gurr, London) for 25 min. at 37°. After further washing, water was removed by careful blotting and the preparations observed directly through immersion oil by bright field microscopy.

Fluorescence with acridine orange technique. One drop of aqueous 1/5000 acridine orange (G. T. Gurr, London) and one drop of sodium hydroxide + acetic acid buffer (0·6 N-acetate, pH 4·5) were placed on the slide for a few seconds and then gently ashed off with a further quantity of pH 4·5 buffer. A coverslip was placed over the wet preparation and the nuclei observed by fluorescence microscopy. The fluorescence of some preparations faded fairly rapidly under illumination so that frequent changes of field were necessary.

Acriflavine phase-contrast technique. The unfixed smear was stained for 1 min. with a drop of 0·01% (w/v) acriflavine (British Drug Houses Ltd.), gently washed,

mounted in distilled water under a coverslip and observed by phase-contrast microscopy.

Polyvinylpyrrolidone phase-contrast technique. The unfixed smear was mounted in 25 % (w/v) polyvinylpyrrolidone (PVP) in 0·5 % NaCl solution and observed through a coverslip by phase-contrast microscopy.

Microscopy. The same microscope (Cooke 2000 binocular) was used for all observations. For bright field and fluorescence microscopy a bright-field condenser of N.A. 1·0 and ×95 objective of N.A. 1·3 were used. For fluorescence microscopy the primary filtration was by 4 cm. of a 2 % ammoniacal copper sulphate solution or two thicknesses of Ilford 304 filter, and the secondary (eyepiece) filters were Ilford 110 minus blue. The light source was a high-pressure mercury vapour lamp.

Phase-contrast observations were made with a condenser of N.A. 1·0 and ×95 phase objective of N.A. 1·3. The combination of eyepieces and binocular body gave an equivalent magnification of ×15.

Scoring procedure. Each preparation was scored independently by two observers. Several fields were scanned with the intention of scoring every bacterium within each field. Bacteria were recorded as having 1, 2, 3, 4 or more nuclei or as being unscorable and each observer scored about 230 bacteria per preparation. The number of unscorable bacteria in the first 200 observed was noted and the statistical analyses were based on the first 200 scorable bacteria recorded by each observer. The results were analysed for differences between treatments and between observers.

RESULTS

There were relatively few unscorable bacteria and their omission from the analyses is unlikely to have contributed any significant bias to the results. It is clear from the data in Tables 1–4 that the four methods gave similar distributions of bacteria with

Table 1. *Nuclear counts of a population of Escherichia coli* WP *2 (1st series) by two observers using four different methods*

Method*	Observer	Proportion of unscorable bacteria (%)	Number out of 200 scorable bacteria with n nuclei				Mean nuclear number	
			$n = 1$	2	3	4		
AF	BAB	0·5	110	89	1	0	1·45 ± 0·04	1·43 ± 0·02
	RJM	5·5	131	67	1	1	1·36 ± 0·04	
AO	BAB	8·5	109	90	1	0	1·46 ± 0·04	
	RJM	3	99	100	1	0	1·51 ± 0·04	
Giemsa	BAB	5	126	73	1	0	1·38 ± 0·03	
	RJM	1·5	119	81	0	0	1·41 ± 0·03	
PVP	BAB	5·5	97	103	0	0	1·51 ± 0·04	
	RJM	0	124	75	1	0	1·39 ± 0·04	

* AF = acriflavine phase-contrast; AO = acridine orange fluorescence; PVP = polyvinylpyrrolidone.

respect to nuclear number. In all except one instance, however, such differences as were observed were significant by the χ^2 test (Table 5). With the Giemsa and fluorescence with acridine orange techniques the observers did not differ

significantly in 7 out of 8 estimations; this is probably a reflexion of the clarity of the microscopic picture obtained with these techniques. The phase-contrast acriflavine and polyvinylpyrrolidone (PVP) techniques yielded preparations which were estimated differently by observers in 6 out of 8 cases, a result which reflects the subjective difficulty in scoring which was especially noticeable with PVP preparations.

Table 2. *Nuclear counts of a population of Escherichia coli* WP 2 *(2nd series) by two observers using four different methods*

Method*	Observer	Proportion of unscorable bacteria (%)	Number out of 200 scorable bacteria with n nuclei: $n=1$	2	3	4	Mean nuclear number	
AF	BAB	5·5	56	136	7	1	1·76 ± 0·04	1·66 ± 0·03
	JL	9	103	79	18	0	1·58 ± 0·05	
AO	BAB	1	70	128	2	0	1·66 ± 0·03	
	JL	0	87	111	2	0	1·58 ± 0·04	
Giemsa	BAB	0·5	67	129	2	2	1·69 ± 0·04	
	JL	7	89	107	4	0	1·58 ± 0·04	
PVP	BAB	9	62	133	4	1	1·72 ± 0·04	
	JL	11	68	130	2	0	1·67 ± 0·03	

* As in Table 1.

Differences which are apparent in the distribution analyses are much less obvious in the mean nuclear numbers. When the latter are considered as a sample of a population centred around a mean value, the most probable overall mean value can be calculated and also the distribution of the individual estimations. If, for a given strain of bacteria, the 'true' mean nuclear number is m, and the mean nuclear numbers estimated by different observers and different methods are regarded as random observations from a normally distributed population of possible estimates of m, then an overall estimate of m (with standard error) can be obtained, and also a confidence interval for m given. The results show that a single mean nuclear count, by any observer or method, based on 200 scored bacteria, would 19 times out of 20 estimate m to within about ±7·5% in the case of *Escherichia coli* WP 2 or about ±12% in the case of *Bacillus subtilis* and *E. coli* B. The corresponding 95% confidence limits for the overall estimates of m (from data by both observers and all four methods) were ±3·6% and ±5·7%.

DISCUSSION

It is apparent from the quantitative data that all four methods made visible the same objects within the bacteria. It is fairly certain that the Giemsa and acridine orange fluorescence techniques make visible DNA, and the objects seen with the phase-contrast techniques are known to behave during the division cycle as one would expect of genetic material (Schaechter *et al.* 1962). There is thus little doubt that, in strains in which the nuclear material exists as a discrete body, it is possible to make visible and estimate with reasonable precision the number of such nuclei in a given population.

The easiest to score were Giemsa preparations, where dark nuclei were seen in a

Table 3. *Nuclear counts for Escherichia coli* B *by two observers using four different methods*

Method*	Observer	Proportion of unscorable bacteria (%)	Number out of 200 scorable bacteria with n nuclei												Mean nuclear number	
			n = 1	2	3	4	5	6	7	8	9	10	11	12		
AF	BAB	2	98	92	1	8	0	1	0	0	0	0	0	0	1·62 ± 0·05	1·53 ± 0·03
	RD	1·5	126	52	14	5	2	0	0	1	0	0	0	0	1·55 ± 0·07	
AO	BAB	1	140	52	1	5	0	1	0	1	0	0	0	0	1·41 ± 0·06	
	RD	0	136	51	6	4	1	1	0	0	0	0	0	1	1·47 ± 0·08	
Giemsa	BAB	2	107	80	4	6	0	0	0	1	1	0	0	1	1·66 ± 0·09	
	RD	1	113	76	2	4	0	3	0	1	0	1	0	0	1·61 ± 0·08	
PVP	BAB	5	140	50	6	2	2	0	0	0	0	0	0	0	1·38 ± 0·05	
	RD	0·5	114	78	2	4	0	2	0	0	0	0	0	0	1·52 ± 0·05	

* As in Table 1.

Table 4. *Nuclear counts for Bacillus subtilis by two observers using four different methods*

Method*	Observer	Proportion of unscorable bacteria (%)	Number of 200 scorable bacteria with n nuclei																Mean nuclear number	
			n = 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16		
AF	BAB	6·5	38	59	10	58	10	9	4	8	0	1	0	1	0	0	0	2	3·34 ± 0·17	3·59 ± 0·08
	RJM	2	45	51	15	40	10	13	5	10	4	1	1	3	2	0	0	0	3·60 ± 0·18	
AO	BAB	4	8	75	17	55	7	12	8	13	0	1	0	1	0	1	1	1	3·81 ± 0·17	
	RJM	0·5	25	59	24	57	12	13	0	4	5	0	0	0	0	1	0	0	3·36 ± 0·14	
Giemsa	BAB	1·5	24	56	14	72	10	9	4	8	1	1	1	0	0	0	0	0	3·46 ± 0·06	
	RJM	0	24	26	22	67	21	18	6	5	1	0	0	0	0	0	0	0	3·67 ± 0·12	
PVP	BAB	4·5	15	62	17	75	9	11	4	4	0	1	1	0	1	0	0	0	3·48 ± 0·13	
	RJM	2·5	19	40	28	51	16	18	8	10	3	3	3	1	0	0	0	0	4·06 ± 0·16	

* As in Table 1.

Table 5. *Probability that the differences in observed distributions are due to chance*

Series	Between observers AF (%)	Between observers AO (%)	Between observers Giemsa (%)	Between observers PVP (%)	Between methods BAB (%)	Between methods Other observer (%)
Escherichia coli WP 2 (1st series)	5	> 5	> 5	1	5	1
E. coli WP 2 (2nd series)	0·1	> 5	> 5	> 5	> 5	0·1
E. coli B	0·1	> 5	> 5	1	0·1	0·1
Bacillus subtilis	> 5	0·1	> 5	0·1	2·5	0·1

pink cytoplasm. The technique was tedious, however, and very occasionally the results were so poor that scoring was impossible. The acridine orange fluorescent preparations were readily made and fairly easy to score once dark adaptation of the eyes had been achieved (usually by means of a velvet hood). Theoretically, resolution with fluorescent light should be slightly inferior to that with transmitted light, and in the former case this might have resulted in the underestimation of newly divided nuclei. There was, however, no indication of this in our results. The acriflavine phase-contrast technique was equally quick and required no dark adaptation. Nuclei appeared as small light areas in a dark cytoplasm and, although this made recognition more difficult than with the reverse contrast, completely unscorable preparations were extremely rare.

The polyvinylpyrrolidone (PVP) preparations were by far the most difficult to score. Not only did the bacteria become detached from the slide but the contrast between nuclear and cytoplasmic regions was not great. In the published photographs of Schaechter *et al.* (1962) the image had been reversed and the contrast considerably enhanced by photographic means. Other workers had reported that the concentration of PVP (or gelatin) was critical but we did not find this to be so. The PVP (or gelatin) method has the important advantage that it is the only one which permits the observation of nuclei in living organisms.

It is clear from the data given here that there is little to choose between the four methods as far as the quantitative estimation of nuclear numbers is concerned. Other factors might, therefore, determine the most suitable method for a given system in any future work. The differences between individual observers could probably have been decreased by scoring nuclei from photographs, but the loss of resolution for slightly out of focus nuclei appeared to outweigh the advantages of this procedure.

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The Effect of Chloramphenicol on the Growth of *Scenedesmus quadricauda*

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SUMMARY

The effects of different concentrations of D (−) threo and L (+) threo-chloramphenicol on the growth of *Scenedesmus quadricauda* have been studied. The results indicate: (*a*) both isomers caused an increase in the total population largely independent of concentration; (*b*) at concentrations between 100–200 μg./ml. both isomers gave a secondary increase in growth after a secondary lag period of 4–6 days; (*c*) both isomers inhibited the growth rate at concentrations comparable with those which inhibit bacterial growth and protein synthesis. The L (+) threo isomer was twice as effective as the D (−) threo isomer, though it did not give any inhibition below 22 μg./ml. (*d*) above 50 μg./ml. the D (−) threo isomer gave a significantly greater lag period than below this concentration. The L (+) threo isomer did not affect the lag period below 25 μg./ml., but above this concentration, an increase in the concentration lengthened the lag period at the same rate as with the D (−) threo isomer above 50 μg./ml.

INTRODUCTION

Chloramphenicol has been shown to inhibit the growth of bacteria, and its mode of action is thought to be by inhibition of protein synthesis (Brock, 1961). Lacks & Gros (1960) found that the initial rate of incorporation of radioactive amino acids into RNA in *Escherichia coli* was decreased by 80 % with, for instance, chloramphenicol 20 μg./ml. Concentrations of this order do not affect respiration. In higher plant tissues, chloramphenicol inhibits other physiological processes, e.g. ion accumulation in beetroot slices and carrot roots (Sutcliffe, 1960), and calcium binding in maize mitochondria (Hanson & Hodges, 1963), though at concentrations 40–100 times greater than those which inhibit protein synthesis in bacteria. Although these inhibitions have been ascribed to inhibition of protein synthesis and Jacoby & Sutcliffe (1962) have shown that protein synthesis is inhibited in beetroot slices at these concentrations, Hanson & Hodges (1963) and Stoner, Hodges & Hanson (1964) showed that the calcium binding in maize mitochondria was inhibited under conditions where no net protein synthesis was to be expected. They further noted that the effect of chloramphenicol was much like that of the uncoupling agent 2,4-dinitrophenol, and showed that chloramphenicol at 0·8–1·6 mg./ml., decreased the P/O ratio very considerably. They thought that observations such as those of Jacoby & Sutcliffe (1962) might be secondary effects due primarily to the uncoupling of oxidative phosphorylation. Ellis (1963) reported that when precautions were taken to overcome the inhibition of amino acid uptake in beetroot slices, there was no chloramphenicol inhibition of L-leucine and L-threonine incorporation

into the trichloroacetic acid-insoluble fraction, although salt uptake was still inhibited.

The effect of chloramphenicol on the growth of micro-organisms other than bacteria has been somewhat neglected, because most of them appeared to be insensitive, though this may have been because in some cases at least, too small concentrations were used. Mager (1960) reported that the growth of *Tetrahymena pyriformis* was 'considerably delayed or completely arrested at 25–150 μg./ml.' without saying whether this referred to the lag phase, or to the growth rate.

Vazquez (1964) stated that no effect of chloramphenicol has been obtained on the growth of green plants but Tamiya, Morimura & Yokota (1962) showed that the growth rate of the green alga *Chlorella ellipsoidea* was affected by chloramphenicol 300–3000 μg./ml. but not by 30 μg./ml. Although growth was completely suppressed at the highest concentration, cell division occurred once, at the same time as in cells in a control culture, but gave extremely small daughter cells which were incapable of further growth when transferred to the normal culture medium. Kumar (1963) found that the maximum concentration of chloramphenicol which permitted growth of the blue-green alga *Anacystis nidulans* was 6 μg./ml.; over the range 1–6 μg./ml. there was no effect on the growth rate, but with higher chloramphenicol concentrations there was an increase in the lag period. However, he did not prolong incubation of his cultures at the higher concentrations beyond 12 days.

Thus the green alga *Chlorella ellipsoidea* appears to be affected by chloramphenicol at concentrations similar to those which affect the higher plants, but *Anacystis nidulans* is as sensitive as the bacteria. It may be significant that Echlin (1964) said that this alga showed, under the electron microscope, structural features which previously had only been associated with bacteria. The experiments reported below were done as a preliminary to other work on the effect of chloramphenicol on metabolic processes in *Scenedesmus quadricauda*; they showed that this green alga has a sensitivity to chloramphenicol of the same order as that of bacteria, and considerably higher than that of Chlorella from which, systematically, it is not far removed.

METHODS

Organism. Scenedesmus quadricauda (Turp.) Breb., originally isolated by Professor W. Rodhe (Uppsala, Sweden) and obtained from the culture collection of algae at the Plant Physiological Institute, University of Göttingen, Western Germany, as strain 276/4C, through the courtesy of Professor A. Pirson.

Cultivation. The culture solution was Österlind's (1949) solution C, which contains sodium carbonate as a CO_2 source; 25 ml. culture medium was contained in 100 ml. conical flasks. The cultures were grown at 25° under continuous fluorescent lighting from below. The light intensity was 4500 lux at the level of the culture, which was a light-saturating intensity for growth under these conditions. The cultures were shaken twice a day.

Chemicals. D (−) threo-chloramphenicol was obtained from Parke Davis and Co. (Hounslow, Middlesex, England) who also provided the sample of L (+) threo-chloramphenicol. The chloramphenicol solutions were sterilized by Seitz filtration, and added to culture media after these had been autoclaved.

Coenobial counts. A sample (0·25 ml.) was removed on each occasion for counting coenobia in a haemocytometer. The small percentage of coenobia with fewer or more cells than four were adjusted to four-celled coenobia.

Lag periods. The coenobial counts were plotted as log. coenobial numbers/mm.^{3} against time, and the primary lag periods were calculated to the nearest 0·25 day from the time of inoculation to the intercept of the calculated logarithmic growth line on the mean lag population. The secondary lag periods were calculated in a similar way from the intercepts of the two logarithmic growth lines on the mean stationary population.

Growth rates. Both primary and secondary growth rates were calculated as the coefficient b in the formula: $\log. N = a+bt$, which is the regression line of log. coenobial numbers/unit volume (N) on time (t in days). They are thus estimates of the specific growth rates.

RESULTS

Effect of D (−) *threo-chloramphenicol on growth*

A series of cultures were set up at D (−) chloramphenicol concentrations from 0·1–200 μg./ml. Illustrative curves are shown in Fig. 1, and the relevant data extracted from the whole series in Table 1. First of all, an effect can be seen on the total population. There were no statistically significant differences between the total

Table 1. *Effect of* D (−) *threo-chloramphenicol concentration on growth of Scenedesmus quadricauda*

Chloramphenicol concentration (μg./ml.)	Primary growth rate*	Secondary growth rate*	Primary lag period (days)	Secondary lag period (days)	Primary maximum population (coenobia/mm.^{3})	Secondary maximum population (coenobia/mm.^{3})
0	0·59	.	0	.	596 ± 37	.
0·1	0·51	.	0	.	752 ± 60	.
1	0·37	.	1·5	.	768 ± 69	.
5	0·32	.	5·0	.	758 ± 44	.
10	0·30	.	5·5	.	742 ± 39	.
25	0·26	.	7·5	.	692 ± 36	.
50	0·20	.	9·0	.	717 ± 41	.
100	0·17	0·06	15·75	4·5	698 ± 43	1638 ± 72
150	0·14	0·05	18·5	4·0	734 ± 42	1748 ± 98
200	0·14	0·04	23·25	5·75	867 ± 51	1642 ± 85

* Growth rate calculated as coefficient b in formula $\log N = a+bt$, which is regression line of log. coenobial numbers/unit volume (N) on time (t in days).

populations from chloramphenicol derivative 0·1–150 μg./ml., but all these showed statistically significant increases over the control. The culture at 200 μg./ml. showed a further statistically significant increase over the 150 μg./ml. culture. Moreover, at 100 μg./ml. and above, after a second lag period, further growth took place, though at a much lower rate than during the original increase, giving a final population which was roughly double that of the first stationary phase. There was also a change in behaviour in the duration of the primary lag period above 50 μg./ml. Above this concentration the lag period was considerably shorter than would have been expected from the values at the lower concentrations. When the length of the

lag period was plotted against log. chloramphenicol concentration (Fig. 2) there was a clear discontinuity. Both parts of the curve showed high correlation coefficients between the duration of the lag and log. chloramphenicol concentration.

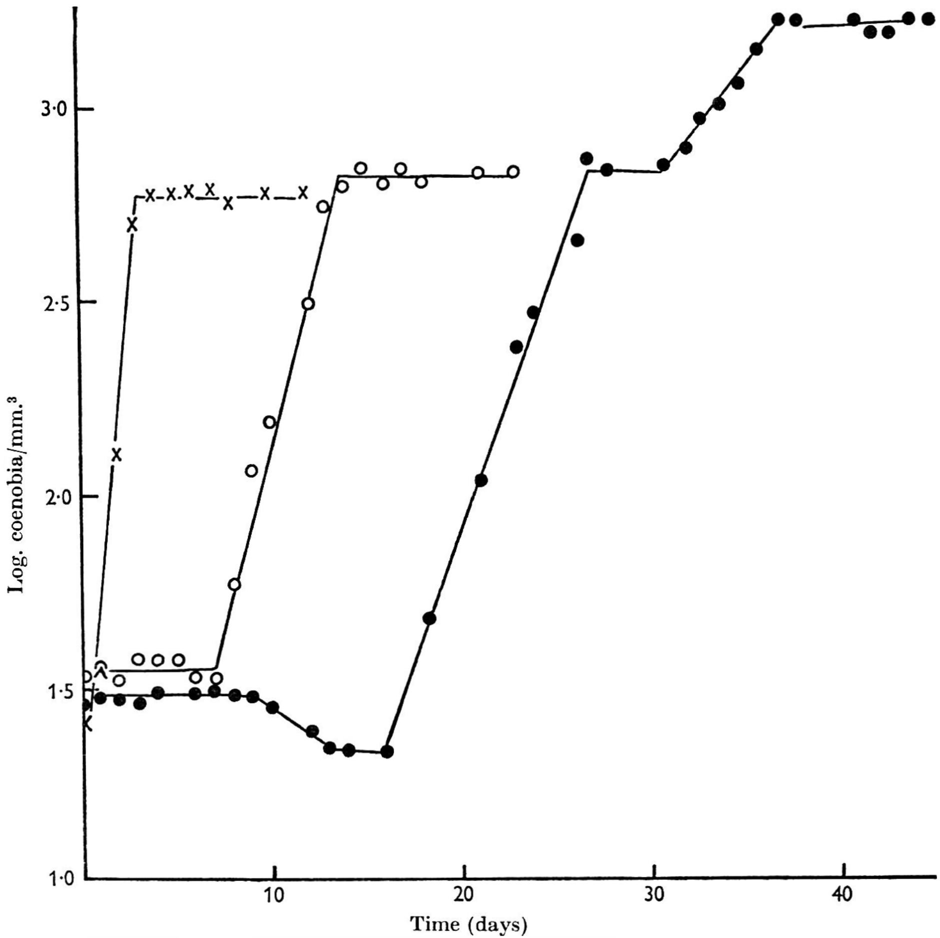

Fig. 1. Effect of D (−) threo-chloramphenicol at 25 and 100 μg./ml. on growth (coenobia/mm.³). of *Scenedesmus quadricauda*. Control, crosses; 25 μg./ml., open circles; 100 μg./ml., closed circles.

The two regression lines of lag period on log. chloramphenicol concentration have been calculated:

Below 50 μg./ml.:
  correlation coefficient $+0{\cdot}97$ (significant at $P = 0{\cdot}01$),
  regression line $y = 3{\cdot}36x + 2{\cdot}64$;

above 50 μg./ml.:
  correlation coefficient $+0{\cdot}95$ (significant at $P = 0{\cdot}05$),
  regression line $y = 25{\cdot}5x - 34{\cdot}85$.

The difference between the two coefficients of x is significant at $P = 0{\cdot}001$.

Correlated with these differences in overall physiological behaviour at higher and lower D (–) chloramphenicol concentrations were differences in the appearances of the organisms. At 50 μg./ml. and below the D(–) chloramphenicol-treated organisms were normal in appearance and size. At 100 μg./ml. and above coenobia during logarithmic growth tended to clump in groups of up to 16 coenobia. These groups were frequently in fours and contained an empty coenobium, suggesting that after liberation the daughter coenobia were prevented from dispersion, possibly by an excessive production of mucilage.

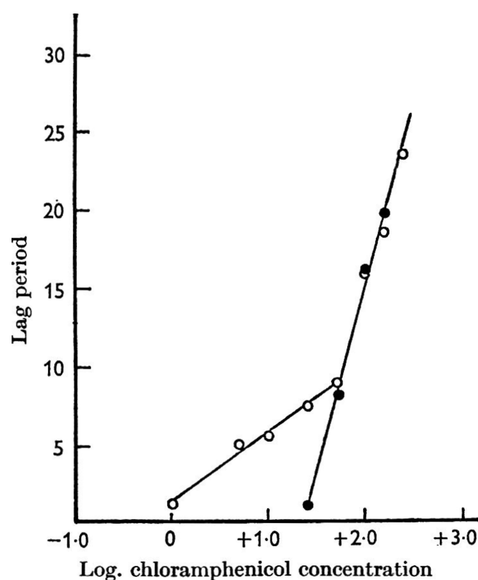

Fig. 2

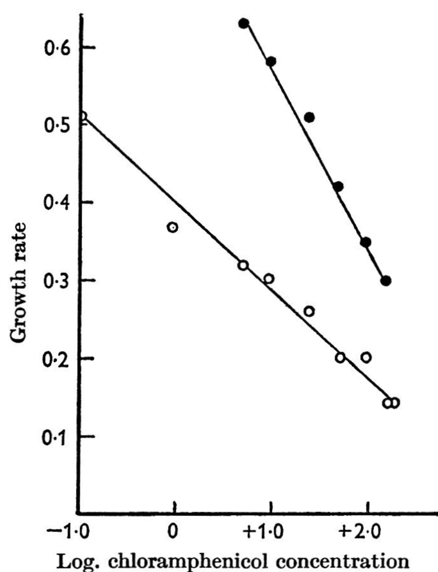

Fig. 3

Fig. 2. Relationship of lag period and chloramphenicol derivative concentration. Open symbols D (–) threo-chloramphenicol; closed symbols L (+) threo-chloramphenicol. No lag period observed below 1 μg./ml. D (–) threo-ehloramphenicol and 25 μg./ml. L (+) threo-chloramphenicol.

Fig. 3. Relationship of growth rate and chloramphenicol derivative concentration. Open symbols D (–) threo-chloramphenicol; closed symbols L (+) threo-chloramphenicol. Control growth rate with D (–) threo-chloramphenicol = 0·59; control growth rate with L (+) threo-chloramphenicol = 0·62.

During the lag phase at these higher concentrations all the cells slowly became disorganized. The extent to which disorganization took place was very variable from cell to cell, even within the same coenobium. The first signs were a contraction of the protoplasts, disappearance of chlorophyll and disintegration of the chloroplasts. However, about 3 days before logarithmic growth began, by which time all the cells were disorganized to a greater or lesser extent (30–60 % completely empty) signs of rejuvenescence appeared in some of the cells. Single cells in a coenobium (more than two in one coenobium were not seen) showed an expansion of the protoplast and a redevelopment of chlorophyll. These rejuvenated cells then began to multiply. These disorganized coenobia were included in the coenobial counts during the lag period, but not during logarithmic growth. Completely empty coenobia

were not counted; this accounts for the apparent decrease in numbers towards the end of the primary lag period with D (–) chloramphenicol 150 μg./ml. in Fig. 1.

The growth rate was also affected by the chloramphenicol concentration, but did not show a discontinuity (Fig. 3). Over the whole range of concentration there was a very high negative correlation between the growth rate and the log. chloramphenicol concentration (–0·99, significant at $P = 0{\cdot}01$). The regression line of growth rate on log. chloramphenicol concentration was determined as:

$$y = 0{\cdot}40 - 0{\cdot}11x.$$

Effect of D (–) threo-chloramphenicol on the growth of coenobia previously grown in D (–) threo-chloramphenicol 25 μg./ml.

A series was set up with inocula of coenobia which had reached the stationary phase of growth in D (–) chloramphenicol 25 μg./ml. There were no statistically significant differences in behaviour as compared with the previous experiment beyond the fact that the control (i.e. inoculum grown in D (–) chloramphenicol 25 μg./ml. but inoculated into chloramphenicol-free medium) produced the same maximum population (720 ± 49) as did chloramphenicol-treated organisms. The growth rate in this flask was also lower than in the first experiment (0·49). These results are consistent with the effect to be expected from the chloramphenicol carried over in the inoculum. This, if none had been absorbed by the organisms in the original culture, would have given a concentration of chloramphenicol 0·5 μg./ml. In practice it would be somewhat lower than this. There was thus no obvious immediate adaptation of the organism to tolerate chloramphenicol.

Effect of L (+) threo-chloramphenicol on growth

The L (+) threo isomer of chloramphenicol is not antibacterial, and is much less active than the D (–) threo isomer as an inhibitor of amino acid incorporation into protein (Rendi & Ochoa, 1962). Ellis (1963) however, found it to be 65–115% as effective as the D (–) threo isomer in inhibiting sulphate uptake of plant tissue slices, the degree of inhibition depending upon the species and chloramphenicol concentration. A similar series to the first experiment was done with L (+) threo-chloramphenicol. Typical results are shown in Fig. 4, and the extracted data in Table 2. The general effect was much the same as with D (–) threo-chloramphenicol, though the L (+) isomer did not manifest its effects at such low concentrations, and there were significant quantitative differences.

Up to L (+) isomer 50 μg./ml. the effect on the maximum coenobial population is the same as with the D (–) isomer, and there were no statistical differences between the maximum populations at corresponding concentrations up to this value. At 100 and 150 μg./ml. the primary population was significantly increased to an even greater extent than with D (–) threo-chloramphenicol 200 μg./ml. These two highest concentrations also show the same secondary growth effect as was observed with the D (–) threo isomer at these concentrations and to the same extent (a doubling of the population), but the final total population was very much greater with the L (+) threo isomer.

A plot of the lag period against log. concentration (Fig. 2) did not show the discontinuity observed with the D (–) isomer, and no lag was observed at 10 μg./ml.

or below. Over the range 25–150 μg./ml. the correlation coefficient between lag duration and log. chloramphenicol concentration was +0·99, significant at $P = 0{\cdot}001$, and the regression line of lag period on log. chloramphenicol concentration was: $y = 23{\cdot}5x - 31{\cdot}47$. There were no statistically significant differences between the

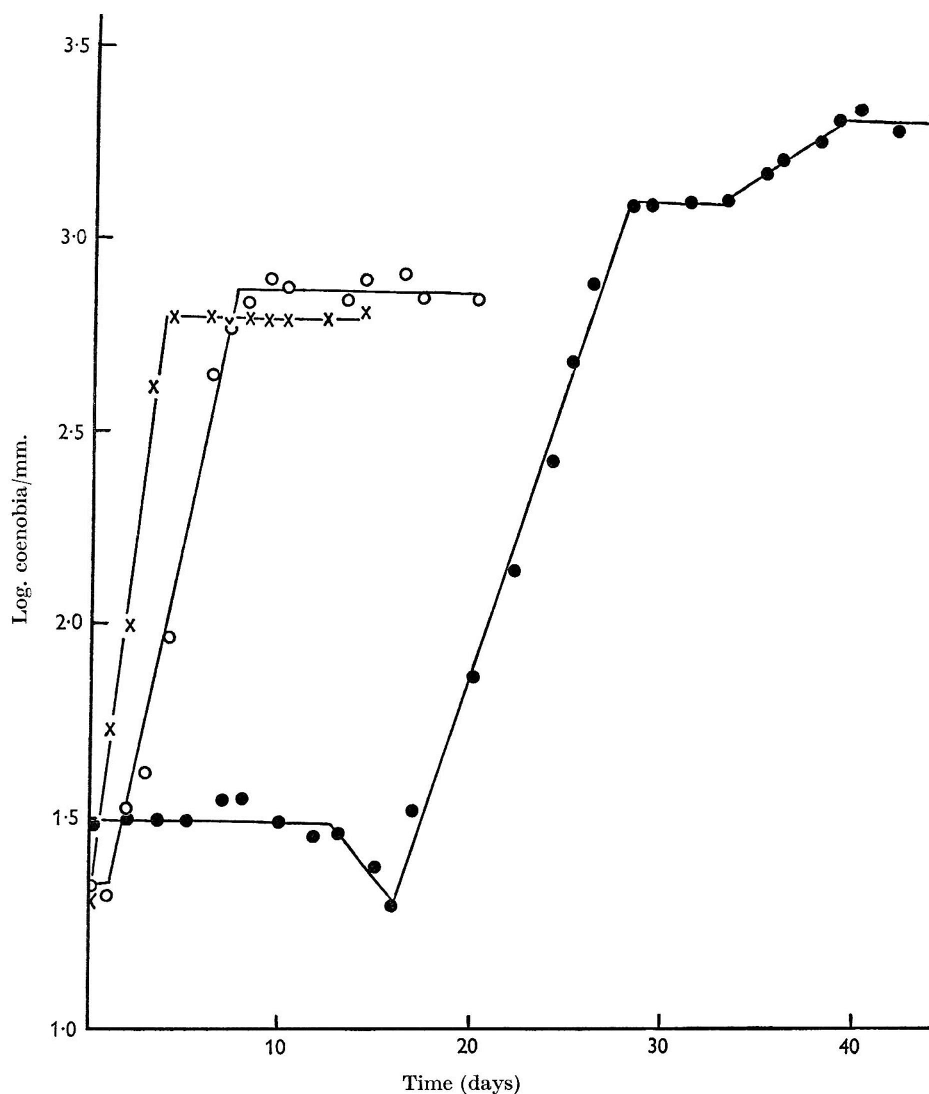

Fig. 4. Effect of L (+) threo-chloramphenicol at 25 and 100 μg./ml. on growth (coenobia/mm.[3]) of *Scenedesmus quadriacauda*. Control, crosses; 25 μg./ml., open circles; 100 μg./ml., closed circles.

coefficients of x or the constants in the regression lines for the L (+) isomer and the upper range of the D (−) isomer. We may therefore conclude that the two sets of points fall on the same line, and that both isomers affect the lag period through their effect on the same process and to the same extent. However, the D (−) isomer also affected the lag period through another process, which was unaffected by the L (+)

isomer. The degree of inhibition of this second process by the D (−) isomer was relatively greater than the inhibition of the first at concentrations lower than 50 μg./ml., but the relative inhibitions were reversed above this concentration.

The L (+) isomer had similar effects to the D (−) isomer on the cellular morphology at 50 μg./ml. and above.

Table 2. *Effect of* L (+) *threo-chloramphenicol concentration on growth of Scenedesmus quadricauda*

Chloramphenicol concentration (μg./ml.)	Primary growth rate*	Secondary growth rate*	Primary lag period (days)	Secondary lag period (days)	Primary maximum population (coenobia/ mm.^{3})	Secondary maximum population (coenobia/ mm.^{3})
0	0·62	.	0	.	648 ± 31	.
1	0·61	.	0	.	752 ± 45	.
5	0·63	.	0	.	709 ± 49	.
10	0·58	.	0	.	774 ± 42	.
25	0·51	.	1·25	.	754 ± 47	.
50	0·42	.	8·0	.	748 ± 39	.
100	0·35	0·07	16·25	4·5	1292 ± 53	2252 ± 61
150	0·30	0·06	19·75	5·25	1304 ± 71	2212 ± 159

* Growth rate calculated as coefficient b in formula $\log N = a + bt$, which is regression line of log. coenobial numbers/unit volume (N) on time (t in days).

The effect of L (+) threo-chloramphenicol on the growth rate showed a very high negative correlation between growth rate and the log. chloramphenicol concentration (−0·999; Fig. 3); and the regression line of growth rate on log. chloramphenicol concentration was: $y = 0{\cdot}81 - 0{\cdot}23x$. The coefficient of x for the L (+) isomer was twice that of the corresponding coefficient for the D (−) isomer; the difference was statistically significant ($P = 0{\cdot}01$). The two regression lines cut the ordinate at $x = 3{\cdot}63$ (D (−)) and 3·53 (L (+)). The standard error of estimate of x for the D (−) isomer was 1·8 %. Since the value of x when $y = 0$ for the L (+) isomer differed from the value for the D (−) isomer by less than twice this standard error, we can conclude that if both regression lines were extrapolated to the ordinate, they would cut it at the same point. In other words the concentrations of both isomers necessary for complete theoretical inhibition are the same. It seems likely therefore, that they both act on the same process, but at different rates. The L (+) isomer was twice as effective an inhibitor as the D (−) though it only began to inhibit at a higher concentration, possibly through differences in permeability.

DISCUSSION

Although *Scenedesmus quadricauda* is an autotrophic organism, the kinetics of its growth can be treated like those of the bacteria (Hinshelwood, 1946). Its considerably longer growth period, though, does not make it such a convenient organism for extended studies of adaptation. The increase in total population on treatment with the chloramphenicol derivatives suggests that nutrient exhaustion was not the factor causing the cessation of normal logarithmic growth. The limiting factor appears to be the pH of the medium, a pH of 12 coinciding with the cessation of growth in

normal cultures. The cessation of growth in chloramphenicol cultures below 100 μg./ml. was found to cease when the pH was between 10 and 11. By lowering the pH of a control culture in the stationary phase it was found possible to increase the population from 581 ± 45 and 780 ± 53. It is possible, therefore, to account for the increase in population with chloramphenicol to a delay in the attainment of an adverse pH. This might be effected through differences in the relative rates of inhibition of cation and anion uptake. Hinshelwood (1946, p. 72) has noted that the influence of pH upon total population in bacterial cultures consists largely of changing the limiting factor from exhaustion of foodstuff to accumulation of inhibitors. We have no experimental evidence for the occurrence of any external inhibitors beyond the hydroxyl ion (if in fact it is this aspect of pH which limits growth).

The pattern of response of total population at 100 μg./ml. and above is reminiscent of the phenomenon of diauxie (Monod, 1942). However, in the case of Scenedesmus there does not appear to be a second source of nutrition unless it be the chloramphenicol itself, which appears unlikely. Moreover, the phenomenon has appeared in some experiments without chloramphenicol, under growth-limiting light conditions, and the phenomenon merits further investigation. We may note, however, that under certain experimental conditions (which are not obtained in these chloramphenicol experiments), *Scenedesmus* cells are capable of releasing considerable amounts of carbohydrate into the medium, which could conceivably act as an energy-source.

The possibility that this secondary increase is an artefact due to the prolonged sampling, and consequent lowering of the culture volume, was considered, but since no secondary increase was observed after the same number of samples had been taken from a 25 μg./ml. culture, and since it took place later with the 200 μg./ml. culture than with the 100 μg./ml. culture, though both were sampled on the same occasions, the effect is considered real.

Woof & Hinshelwood (1961) noted a discontinuity in the shape of the lag/chloramphenicol concentration curve with 'Bacterium lactis aerogenes' (*Aerobacter aerogenes*). They favoured the explanation that chloramphenicol affected two different functions of the organisms rather than that there was a small number of pre-existing mutants resistant to the low drug concentrations but sensitive to high ones. The form of the lag/concentration curve is thought to favour a similar explanation for *Scenedesmus quadricauda*, with the additional support that only one of these functions was affected by the L (+) isomer used. Similar conclusions can be drawn from the observations of the cellular changes. No cellular disintegration was observed at concentrations below the discontinuity, but were found in all cells above, showing that there was a distinct difference in effect in the two ranges. The cells which recovered from degeneration were those statistically favoured members of the population involved in a competition between 'adaptive' and lethal processes.

Only one effect on the growth rate was observed, and the L (+) isomer was twice as effective an inhibitor of this as the D (−) isomer. This makes it unlikely that the growth rate was limited in the treated cells by either of the processes which limit the lag period in the presence of chloramphenicol derivative.

There is a considerable difference between various organisms and tissues in the concentration at which chloramphenicol inhibitions occur. The responses of

Scenedesmus quadricauda occur at the same order of concentration as for bacteria, as opposed to higher plant tissues and the only other green alga which has been investigated, Chlorella. These differences might be due to differences in permeability to chloramphenicol in the two groups, which might operate through differences in the proteins or lipids of the plasma membrane, or be due to differences in the site of chloramphenicol binding. Vazquez (1964) showed considerable differences in the latter as between sensitive and resistant organisms at low chloramphenicol concentrations.

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Uptake of ^{3}H-Griseofulvin by Micro-organisms and its Correlation with Sensitivity to Griseofulvin

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SUMMARY

A correlation was observed between the sensitivity of different micro-organisms to griseofulvin and their ability to take up the antibiotic, especially into their nucleic acid and protein fractions. The insensitive yeasts *Candida albicans* and *Saccharomyces cerevisiae* and the bacterium *Escherichia coli* did not bind an appreciable amount of [4-methoxy-^{3}H] griseofulvin. The poorly sensitive filamentous fungi *Aspergillus niger* and *Neurospora crassa* accumulated a considerable quantity of antibiotic, mostly in the water-soluble pool. This was in contrast to the highly sensitive dermatophytes *Microsporum gypseum*, *Trichophyton mentagrophytes* and *T. persicolor* in which the nucleic acid and protein fraction contained about half of the total bound griseofulvin (as relatively stable complexes). It is proposed that uptake of griseofulvin is essential for antibiotic action and that the degree of sensitivity shown by an organism is dependent upon the tendency of its macromolecules to complex with the accumulated griseofulvin. Griseofulvin was not degraded by the insensitive or the poorly sensitive organisms tested. Metabolic products of griseofulvin were detected, however, in culture fluids of the sensitive dermatophytes.

INTRODUCTION

Investigations of the response of different micro-organisms to griseofulvin (Brian, 1949; Roth, Sallman & Blank, 1959; El-Nakeeb, McLellan & Lampen, 1965) have singled out the dermatophytes as the most sensitive group. Other filamentous fungi varied in their sensitivity from moderate (plant pathogens) to poor (saprophytes). Yeasts and bacteria were insensitive. The special sensitivity of the dermatophytes stimulated investigations on the uptake of ^{3}H-griseofulvin by cultures of *Microsporum gypseum* (El-Nakeeb, 1963; El-Nakeeb & Lampen, 1965*a*, *b*). The principal observations may be summarized as follows. (1) The uptake process involved two phases: the first was rapid and independent of viability of the organisms; the second phase was prolonged and controlled by factors which govern active metabolism (e.g. pH value, temperature, metabolic energy supply) and protein synthesis (probably of a transport system) was required. (2) The fungus concentrated griseofulvin intracellularly to a value about 100-fold that of the medium. (3) The radioactivity which remained in the medium was present either as griseofulvin complexed with cell materials or in degradation products of the griseofulvin. (4) The intracellular ^{3}H-griseofulvin was bound to fungal constituents and could be extracted (mostly as complexes) with boiling water, hot trichloroacetic acid and hot NaOH. There was essentially no ^{3}H-label in the nucleic acids and proteins themselves.

Attempts to show uptake of griseofulvin by *Botrytis allii*, *Phycomyces blakeseeanus*, or by *Mucor ramannianus* have been unsuccessful (Abbott & Grove, 1959); but it should be noted that the microspectrophotometric method then used was not sufficiently sensitive to detect small amounts of griseofulvin. Also, these fungi were either poorly sensitive (*P. blakeseeanus*, *M. ramannianus*) or only moderately sensitive (*B. allii*) to griseofulvin. Boothroyd, Napier & Somerfield (1961) stated that about 50 % of the ^{36}Cl-griseofulvin added to *B. allii* cultures could be accounted for in the mycelium. The present study was designed to seek any relationship between the degree of sensitivity of a micro-organism to griseofulvin and the organism's uptake and metabolism of this antibiotic.

METHODS

Organisms and media. The following organisms were used: *Escherichia coli* B, *Candida albicans* 204, *Saccharomyces cerevisiae* LK 2 G 12, *Aspergillus niger* C 116, *Neurospora crassa* A-17, *Trichophyton mentagrophytes* X 8 (two strains: one partially resistant and the corresponding parent sensitive strain), *Trichophyton persicolor* C 654. The sources of the strains, the media and the culture conditions were described earlier (El-Nakeeb *et al.* 1965).

Uptake of griseofulvin. For the filamentous fungi, triplicate series of young rapidly growing cultures (shaken to produce dispersed growth) were prepared. These cultures were generally 48–72 hr old and contained about the equiv. of 3 mg. dry wt. mycelium/10 ml. For each experiment with *Escherichia coli*, *Candida albicans* or *Saccharomyces cerevisiae*, a log phase culture was divided aseptically into three equal volumes. The three series were handled according to the general procedure previously described by (El-Nakeeb & Lampen (1965*a*): microvolumes of a ^{3}H-griseofulvin solution in dimethylsulphoxide were added to the cultures of series A and B; equal volumes of dimethylsulphoxide were put into series C as a control, and all cultures then shaken at 30°. At specified times samples were withdrawn aseptically (or individual cultures were used) for measurement of griseofulvin uptake (series A) or for determination of mycelial weight (series B, C).

Isolation of intracellular radioactive compounds. Organisms were washed twice with ice-cold distilled water and then successively extracted with boiling water (water-soluble pool) cold trichloroacetic acid, hot trichloroacetic acid (nucleic acid fraction) and finally with hot NaOH (protein fraction) under the conditions adopted for *Microsporum gypseum* (El-Nakeeb *et al.* 1965). Chromatography of the extracts was done by the procedure of El-Nakeeb & Lampen, (1965*b*). Radioactivity was estimated by the liquid scintillation method (El-Nakeeb & Lampen, 1965*a*). All values have been corrected for quenching.

RESULTS

Lack of uptake by insensitivie organisms

Escherichia coli, *Candida albicans* and *Saccharomyces cerevisiae* did not take up ^{3}H-griseofulvin to an appreciable extent (Table 1). The small amounts of radioactivity removed by the organisms from the medium (0·4–0·8 % of the radioactivity added) might have been due to non-specific adsorption, since most of the uptake occurred

almost instantaneously at 4° (0 hr uptake, Table 1). Comparable amounts of ^{3}H-griseofulvin were adsorbed by autoclaved cultures of *Microsporum gypseum* (El-Nakeeb & Lampen, 1965*a*). The low uptake of ^{3}H-griseofulvin might result from restricted permeability to the antibiotic or from its rapid degradation by the organisms. The latter possibility can be excluded (at least for *E. coli* and *S. cerevisiae*) since at the end of the growth period (Table 1) about 99 % of the added radioactivity was still present as chloroform-extractable undegraded griseofulvin (determined by chromatographic behaviour). It should be emphasized that the griseofulvin added did not inhibit the growth of these organisms (dry wt. increase) or produce detectable morphological changes.

Table 1. *^{3}H-griseofulvin binding by insensitive organisms*

	Counts/min. × 10^{-3}/10 ml. culture			Total uptake in mycelium	
		Organism			
Time (hr)*	Filtrate	Water extract	Residue	Added ^{3}H-griseofulvin %	µg./equiv. mg. dry wt.
		Escherichia coli			
0	975†	6·0	0·5	0·7	0·44
50	962	6·6	1·0	0·8	0·08
		Candida albicans			
0	1160	4·4	0·5	0·4	0·08
24	1147	5·2	0·9	0·5	0·03
		Saccharomyces cerevisiae			
0	1220	4·7	0·1	0·4	0·04
48	1205	6·3	0·2	0·5	0·02

* The 0 hr sample was taken immediately after addition of 10 µg. ^{3}H-griseofulvin/ml. to a growing culture (see Methods).

† 0 hr value is counts/min. × 10^{-3} added to culture.

Uptake of ^{3}H-griseofulvin by poorly sensitive fungi

The filamentous fungi *Aspergillus niger* and *Neurospora crassa* gradually took up considerable quantities of ^{3}H-griseofulvin, most of which was extractable by hot water (Table 2); the fraction not extracted was relatively small, but it increased gradually with time of incubation. *A. niger* accumulated more radioactivity per unit weight of mycelium and at a faster rate than did *N. crassa*. The significance of this difference is not clear, since griseofulvin 10 µg./ml. had only a slight effect (10–20 % inhibition) on the growth (dry wt. increase) of either organism.

Distribution of ^{3}H-griseofulvin taken up by Aspergillus niger and Neurospora crassa

Mycelium from 6 and 48 hr cultures was extracted with boiling water, cold trichloroacetic acid, hot trichloroacetic acid, and hot NaOH, and the radioactivity in the extracts was estimated (Table 3). The water-soluble fraction contained 86–94 % of the total radioactivity. The rest of the radioactivity was mainly in the protein fraction (hot NaOH extract), with small amounts in the hot or the cold trichloroacetic acid extracts. The distribution of intracellular ^{3}H-griseofulvin

was very similar in the two fungi. It was, however, completely different from the proportions in *Microsporum gypseum*, in which there was a ratio of about 2:1:1 for the griseofulvin in the fractions water-soluble pool: nucleic acid fraction: protein fraction, respectively (El-Nakeeb & Lampen, 1965*b*).

Table 2. *Uptake of griseofulvin by Aspergillus niger and Neurospora crassa*

	Counts/min. × 10^{-3}/10 ml. culture			Total uptake	
		Mycelium			^{3}H-griseofulvin μg./ml. dry wt. mycelium
Time (hr)*	Filtrate	Water extract	Residue	% of added ^{3}H-griseofulvin	
		Aspergillus niger			
0	1310†	37	1	2·9	0·59
3	1076	215	6	16·9	1·69
6	1023	252	8	19·8	1·24
24	1006	278	8	21·8	0·68
48	915	345	16	27·5	0·46
		Neurospora crassa			
0	1773	28	1	1·6	0·23
3	1701	51	2	3·0	0·14
6	1647	112	6	6·6	0·19
24	1511	208	6	12·1	0·16
48	1401	292	11	17·1	0·16

* After addition of ^{3}H-griseofulvin 10 μg./ml. (see Table 1).
† 0 hr value is counts/min. × 10^{-3} added to culture.

Table 3. *Conditions required for extraction of ^{3}H-griseofulvin taken up by Aspergillus niger and Neurospora crassa*

^{3}H-griseofulvin was added at 0 hr to cultures which were treated in parallel with those used for the experiment of Table 2. After incubation for 6 and 48 hr, triplicate cultures were pooled and the mycelium extracted as described in Methods.

		Total extracted	% of total radioactivity in extracts				
Micro-organism	Time (hr)	(counts/ min. × 10^{-3})	Hot water	Cold TCA*	Hot TCA*	Hot NaOH	Residue
Aspergillus niger	6	274	93·5	2·1	1·8	2·2	0·3
	48	403	86·8	1·8	1·8	9·3	0·2
Neurospora crassa	6	136	90·3	1·5	1·5	5·9	0·8
	48	342	86·4	1·3	2·4	9·5	0·3

* TCA, Trichloroacetic acid.

Culture fluids. The radioactive material remaining in the culture fluids of *Aspergillus niger* and *Neurospora crassa* appeared to be unaltered griseofulvin, it was extracted by chloroform, and on paper chromatography had an R_F value identical with that of authentic griseofulvin.

Hot water extract. With *Aspergillus niger* and *Neurospora crassa* about 90–95 % of the radioactivity was present as free griseofulvin. The remaining 5–10 % stayed at the origin of the chromatogram. However, after the material had been extracted

from the paper, incubated with an excess of unlabelled griseofulvin and re-chromatographed, the radioactivity now moved with the added griseofulvin. The hot water extract fraction probaby represented griseofulvin which was complexed reversibly with fungal constituents in the water-soluble pool.

Nucleic acid fraction. The amount of radioactivity was very small (Table 3), and no attempt was made to determine its nature.

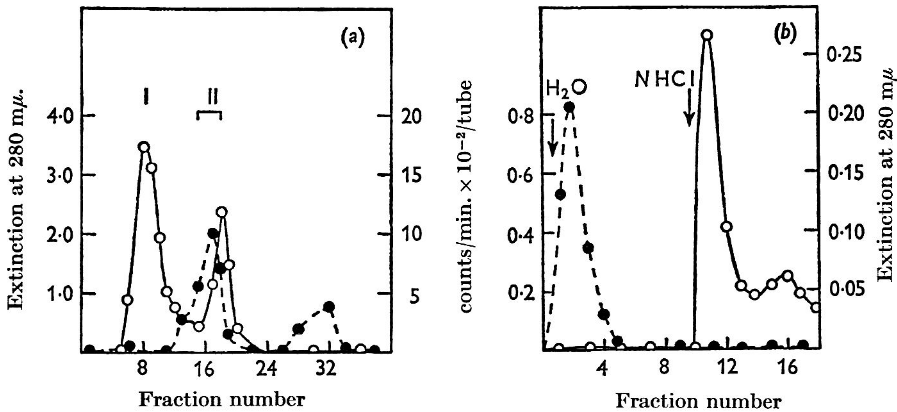

Fig. 1. Complexes of griseofulvin in the protein fraction from *Neurospora crassa*. (*a*) One ml. of the 48 hr NaOH extract (experiment of Table 3) was added to a column of Sephadex G-25 (39 x 0·7 cm.; exclusion volume 8·0 ml.) and eluted with water. One ml. fractions were collected and radioactivity (●—●) and extinction (○—○) determined. (*b*) The fractions of peak II (from *a*) were pooled and 1 ml. of this material added to a column of Dowex-1-chloride (0·4 x 3·0 cm.). The column was washed with water until radioactivity was no longer removed. N-HCl was then passed through the column. The effluent was neutralized and radioactivity and extinction determined. The recovery of radioactivity in the water wash was 101 % of that added to the column.

Protein fraction. The ^{3}H-label in the hot NaOH extracts from *Aspergillus niger* and *Neurospora crassa* was bound to the fungal proteins. An extract prepared 48 hr after the addition of ^{3}H-griseofulvin to a culture of *N. crassa* was subjected to gel filtration on a column of Sephadex G-25 (Fig. 1*a*). The profile of the eluted radioactivity closely followed that of the peptide peak (tubes 15–20) with very little material at the position of free griseofulvin (tubes 27–32). Complete separation of the radioactivity from 280 mμ-absorbing material was achieved on a column of Dowex-1 chloride (Fig, 1*b*). Therefore, the ^{3}H-label had not been incorporated into the amino acid residues and probably was present as complexed griseofulvin.

Uptake of griseofulvin by dermatophytes

As seen previously with *Microsporum gypseum* (El-Nakeeb & Lampen, 1965*a*), there was a substantial uptake of ^{3}H-griseofulvin by the Trichophyton strains, including a partially-resistant culture of *T. mentagrophytes* x 8 (Table 4). The amount of radioactivity concentrated/mg. dry wt. mycelium by *T. mentagrophytes* was much less than with *T. persicolor* (Table 4) or with *M. gypseum*. Even though relatively large amounts of mycelium were present, growth of the sensitive strains of Trichophyton was completely inhibited for at least 24 hr by griseofulvin 10 μg./ml.

The partially resistant strain of *T. mentagrophytes* showed only a 50 % decrease in dry weight gain of mycelium with this concentration of griseofulvin.

Cellular distribution of griseofulvin in dermatophytes. The three Trichophyton cultures (Table 5) accumulated griseofulvin into their nucleic acid and protein

Table 4. *Uptake of ^{3}H-griseofulvin by cultures of Trichophyton persicolor and Trichophyton mentagrophytes* × 8

Time (hr)*	Counts/min. × 10^{-3}/10 ml. culture: Filtrate	Counts/min. × 10^{-3}/10 ml. culture: Mycelium	Total uptake: % of added ^{3}H-griseofulvin	Total uptake: ^{3}H-griseofulvin μg./mg. dry wt. mycelium
	Trichophyton persicolor			
0	165 (172)†	7·9	4·7	0·57
6	148	20·6	12·2	4·1
24	131	26·9	15·8	5·3
72	116	35·8	21·1	2·6
	Sensitive *T. mentagrophytes*			
0	162 (178)†	8·5	4·9	0·26
6	138	34·1	19·8	1·04
24	140	38·0	22·0	1·00
120	110	51·6	30·0	0·80
	Partially resistant *T. mentagrophytes*			
0	1523 (1537)†	12	0·8	0·04
6	1369	162	10·5	0·55
24	1228	293	19·2	0·52
72	1012	508	33·1	0·89

* After addition of ^{3}H-griseofulvin 10 μg./ml. (see Table 1).
† Value in parentheses is counts/min. × 10^{-3} added to culture. Note difference in specific activity of added ^{3}H-griseofulvin.

Table 5. *Distribution of ^{3}H-griseofulvin in mycelium of Trichophyton cultures**

Time (hr)†	% of total uptake present in: Hot H_2O extract	Cold TCA extract	Hot TCA extract	Hot NaOH extract	Cell residue
	Trichophyton persicolor				
6	67	1	1	30	1
24	64	1	7	27	2
72	54	1	18	25	2
	Sensitive *T. mentagrophytes*				
6	53	5	11	28	3
24	49	3	15	31	3
120	41	4	9	42	5
	Partially resistant *T. mentagrophytes*				
6	75	3	6	15	1
24	69	2	7	21	1
72	71	2	7	18	2

* Samples from experiment of Table 4. TCA, Trichloroacetic acid.
† After the addition of ^{3}H-griseofulvin 10 μg./ml.

fractions to a much greater extent than did *Aspergillus niger* and *Neurospora crassa* (Table 3). The distribution of the intracellular radioactivity differed, however, among the several dermatophytes tested. The nucleic acid and protein components of the sensitive culture of *T. mentagrophytes* had more associated activity than did the corresponding constituents of the partially resistant culture (Table 5). This might account in part for the difference in sensitivity of the two cultures to griseofulvin. It may also be significant that each of the Trichophyton cultures bound more radioactivity in the protein fraction than in the nucleic acids (Table 5); *Microsporum gypseum* took up large and equal amounts of activity into the two fractions (El-Nakeeb & Lampen, 1965*b*). Only minor amounts of radioactivity were detected in the cold trichloroacetic acid extracts and in the final cell-wall residues of any of the dermatophytes.

Metabolism of ^{3}*H-griseofulvin by the Trichophyton cultures.* About 80% of the radioactivity which remained in the culture fluid of *Trichophyton mentagrophytes* and *T. persicolor* was in the form of ^{3}H-water or other distillable products. The remaining 20% was extractable with chloroform and was examined by paper chromatography. Three components were found: (1) ^{3}H-griseofulvin (R_F 0·84); (2) a radioactive spot (R_F 0·69); (3) a non-radioactive fluorescent spot (R_F 0·04). In the 6 hr culture about half of this material was free griseofulvin, but after growth for 48 hr essentially all of the radioactivity was in spot (2). It is not certain whether component (2) was a metabolite of griseofulvin or consisted of undegraded griseofulvin bound to cellular products.

The ^{3}H- in the hot-water extracts of the Trichophyton strains (Table 5) appeared to be present as chemically unaltered griseofulvin. At 6 hr, most of the griseofulvin was free (chromatographically) but complexes were present in subsequent extracts. The hot trichloroacetic acid and hot NaOH extracts contained radioactivity firmly bound to the macromolecular constituents. This was inferred from the results of paper chromatography and gel filtration on Sephadex G-25. These data parallel those presented for the corresponding fractions from *Microsporum gypseum* (El-Nakeeb & Lampen, 1965*b*).

DISCUSSION

From the findings reported here two factors appear to be of importance in determining the sensitivity of an organism to griseofulvin: the ability to take up griseofulvin from the medium and the tendency of the cellular nucleic acid and protein to complex with griseofulvin once it has been taken into the cell. For example, the insensitive organisms *Candida albicans, Saccharomyces cerevisiae, Escherichia coli* did not accumulate ^{3}H-griseofulvin (after the initial non-specific adsorption) and did not inactivate it. These organisms may be unable to form a transport system for griseofulvin like that found in *Microsporum gypseum* (El-Nakeeb & Lampen 1965*a*). The second factor is illustrated by the poorly sensitive *Aspergillus niger* and *Neurospora crassa*. These two organisms took up quantities of griseofulvin comparable to those bound by the highly sensitive dermatophytes, but the distribution of accumulated griseofulvin differed greatly between the two groups. *M. gypseum* (El-Nakeeb & Lampen, 1965*b*) and the Trichophyton cultures studied here concentrated about half of the griseofulvin taken up in their protein and nucleic acid fractions. In *A. niger* and *N. crassa*, the corresponding fractions contained not

more than a tenth of the bound griseofulvin and the bulk of the material was extractable with the water-soluble pool. This correlation between high sensitivity to griseofulvin and the concentration of griseofulvin into the nucleic acid and protein fractions is strengthened by the observations on the binding of griseofulvin by RNA from different organisms (El-Nakeeb & Lampen, 1964). Thus, RNA from the sensitive dermatophyte *M. gypseum* formed strong and stable complexes with griseofulvin. Similar preparations from *A. niger* or from yeast had only weak affinity for griseofulvin and any complexes formed were unstable. Although complexing of griseofulvin with nucleic acid and with protein fractions occurred, sensitivity to griseofulvin appears to be more directly correlated with the formation of nucleic acid + griseofulvin complexes. Thus, *A. niger* and *N. crassa* eventually had a moderate amount of griseofulvin complexed with their protein fractions but not with nucleic acid and these organisms are relatively insensitive. Also, the partially-resistant culture of *Trichophyton mentagrophytes* x 8 complexed griseofulvin with its nucleic acid fraction to a lesser extent than did the griseofulvin-sensitive parent strain. The correlation between sensitivity to griseofulvin and its uptake is comparable to that previously reported for penicillin (Eagle, 1954; Rowley, Cooper, Roberts & Smith, 1950) and for streptomycin (Szybalski & Mashima, 1959; Anand, Davis & Armitage, 1960; Hancock, 1962).

Microbial degradation of griseofulvin has been demonstrated only with sensitive organisms; its demethylation was observed by Boothroyd, *et al.* (1961) with the very sensitive *Microsporum canis* and the moderately sensitive *Botrytis allii* and *Cercospora melonis*. We report here degradation by *Microsporum gypseum* and the two Trichophyton species, but not by poorly sensitive *Aspergillus niger* and *Neurospora crassa* cultures. Uptake of griseofulvin (which correlates with sensitivity) may be essential if degradation is to take place at a significant rate.

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CORRIGENDA

In Kerstin Gezelius & Barbara E. Wright (1965)

J. gen. Microbiol. **38**, 309–327

Figs. 7–11, (pp. 321, 323, 324):

For Protein (mμmole/NP/min./mg.) *read* mμmoles NP/min./mg. protein

Page 311, 11 lines from the bottom:

For μMmole NP/min./mg. protein *read* mμmoles NP/min./mg. protein

Page 311, 7 lines from the bottom:

For μMmole Pi/min./mg. protein *read* mμmoles Pi/min./mg. protein

Page 314, lines 5 and 9; page 318 in legend to Fig. 6; page 319, line 5; page 320, lines 13, 14 and 17:

For mμM/min./mg. protein *read* mμmole/min./mg. protein