J. gen. Microbiol. (1964), 37, 157–169
Printed in Great Britain

The Effect of Growth at Elevated Temperatures on Some Heritable Properties of *Staphylococcus aureus*

By J. W. MAY, R. H. HOUGHTON AND C. J. PERRET

Department of Microbiology, University of Western Australia, Medical School, Victoria Square, Perth, Western Australia

(*Received* 9 *April* 1964)

SUMMARY

Populations of a predominantly tetracycline-resistant, penicillinase-positive strain of *Staphylococcus aureus* grown at 43–44° gave rise to progressively increasing proportions of tetracycline-sensitive and penicillinase-negative cocci. The losses did not appear until after the elapse of a number of generations at the elevated temperature, and then apparently proceeded independently, tetracycline resistance being lost more rapidly than the ability to produce penicillinase. Tetracycline-sensitive and penicillinase-negative variants were extremely stable and the growth rates at 44° of the parent strain and the tetracycline-sensitive variants were indistinguishable. Screening for numerous other 'marker' properties revealed no changes under the test conditions. The evidence suggests that tetracycline resistance and penicillinase-forming ability probably depend on the presence of two different plasmids in the cocci; that the replication-rates of the plasmids at elevated temperature are less than that of the cocci; and that the plasmids, once lost, are not spontaneously resynthesized nor, at least for that conferring tetracycline resistance, regained by infection. However, such a hypothesis raises the question of how equilibrium, particularly between the tetracycline-resistant and -sensitive cocci, is maintained in a population growing at 37° and observed to change in one direction but never in the reverse direction.

INTRODUCTION

Strains of *Staphylococcus aureus* which owe their penicillin resistance to the production of penicillinase frequently give rise to penicillin-sensitive variants unable to produce the enzyme (Barber, 1949; Borowski, 1963); this change appears to be favoured by growth of the staphylococci at 44–45° (Bondi, Kornblum & de Saint Phalle, 1953; Fairbrother, Parker & Eaton, 1954). In order to isolate penicillinase-negative variants of penicillinase-positive strains of *S. aureus* for comparison of metabolic properties, a number of suitable strains was therefore grown in a nutrient broth at 44°, with daily subculture. Some of the strains did indeed produce penicillinase-negative variants as time elapsed, and in some instances the change occurred more rapidly than reported by Fairbrother *et al.* (1954). In addition, one strain which initially was also tetracycline-resistant gave rise to apparently 'pure' tetracycline-sensitive populations. The investigations reported here suggest that in this strain of *S. aureus*, penicillinase production and tetracycline resistance, alone among a number of properties tested, are independently determined by the presence of heat-sensitive plasmids.

Vol. 37, *No.* 1 *was issued* 7 *December* 1964

METHODS

Organism. Staphylococcus aureus strain E169, isolated from a clinical source in Western Australia, was used in this investigation. Following three successive single colony pickings, a streak sample was dried in ampoules by the method of Annear (1962); a new ampoule was opened to provide the inoculum for each experiment. Cultures of *S. aureus* E169 produce penicillinase (phenotypically represented by Penase^{+}), and are resistant to penicillin (Pen^{R}) and tetracycline (Tet^{R}). The criteria used for designating the strain Pen^{R} and Tet^{R} were the high survival rates obtained when cultures were plated on medium containing either penicillin G 1 i.u./ml. or tetracycline 3 μg./ml. The phage type of the strain is 52/52A/80/81/KS6.

Growth media. The initial liquid medium was a nutrient broth (NB-1) consisting of 'Oxoid' Nutrient Broth granules, 13 g.; KH_2PO_4, 1·5 g.; K_2HPO_4, 3·5 g.; distilled water to 1000 ml.; autoclaved at 115° for 10 min. A 20% solution of glucose was added immediately before use to give a final concentration of 0·1%. Subsequently, a meat infusion broth (MIB) prepared in the manner described in Mackie & McCartney's *Handbook* (1960) was used. Nutrient agar (NA-1) and meat infusion agar (MIA) were prepared by the addition of 1% 'Oxoid' Ionagar to NB-1 and MIB respectively.

Growth procedure. The bacteria were grown overnight in the liquid medium as a static batch culture at 25°. The following morning 20 ml. of the medium in a plugged 100 ml. conical flask was inoculated from the overnight culture with organisms equivalent to about 0·05 mg. bacterial dry wt./ml. The culture was then incubated at 37° on a hot-room shaker (Kantorowicz, 1951): the population density was maintained as nearly as practicable within the range equivalent to 0·05–0·10 mg. bacterial dry wt./ml. by withdrawal of accurate 10 ml. samples of the culture at suitable intervals, followed by the immediate replacement with 10 ml. of pre-warmed medium. The withdrawn culture samples were killed by the addition of 1 ml. formalin and their extinction measured.

When the extinction measurements showed that the growth rate at 37° had remained constant for three successive doublings, 10 ml. of the culture was rapidly added to 10 ml. of pre-warmed medium contained in a plugged conical flask attached to a shaker in a thermostatically controlled water bath at the elevated temperature (usually within the range 43–44°) and the shaker set at 100 oscillations/min. with an 8 cm. throw. In the initial experiments, the growth temperature was taken as that registered by a mercury thermometer immersed in the bath. When it became obvious that an accurate knowledge of growth temperature was of critical importance, it was measured by means of a plastic-covered thermocouple entering through a side-arm in the growth flask and immersed in the culture. The thermocouple, type F6, was used with an 'Ellab Universal Precision Thermometer', model TE. 3 (Electrolaboratoreit, Copenhagen, Denmark), calibrated against an N.P.L.-certificated standard thermometer. Culture temperatures were found to be constant within ±0·05° throughout an experiment.

The repeated doubling-dilution regime was usually continued at the elevated temperature for about 9 doublings. Of the 10 ml. of culture withdrawn at each dilution stage, 1 ml. was added to 9 ml. of 0·1% peptone water, further diluted in

peptone water, and then plated-out on medium NA-1 or MIA for single-colony pickings; the remaining 9 ml. was formolized and used for extinction measurement.

The plates were incubated at 37° overnight, and 100 or more clearly-isolated colonies picked-off and replicated on a set of plates of 'marker' media in order to detect any changes in the chosen properties. In most of the experiments, the only properties studied were tetracycline susceptibility and penicillinase production. Results of the marker-medium tests were read after further overnight incubation at 37°.

Optical extinction measurements. The extinction of bacterial suspensions was measured in a Hilger 'Spekker' Absorptiometer (Hilger & Watts Ltd., Camden Way, London, N.W. 1, England) with the large 1 cm. light-path cells, the Neutral Grey H 508 filters and the growth medium as blank. Extinction readings were converted to mg. bacterial dry wt./ml. by means of a calibration curve, and then multiplied by the appropriate dilution factor to give a 'cumulative' growth curve.

Cell-wall stain. An agar plate was flooded with the formolized bacterial culture and the excess removed. After drying-in, impression films were prepared by attaching agar blocks to coverslips and immersing overnight in 70% (v/v) ethanol in water containing 1% (v/v) acetic acid. The films were stained by the method of Bisset & Hale (1953) and then mounted over medium type ABP (Valnor Corp., Brooklyn 1, N.Y., U.S.A.). Cocci with deeply stained cross-walls were scored as two cells.

Screening procedure. Colonies to be tested for altered characteristics were picked from the primary plates and suspended in sterile distilled water contained in the wells of a multipoint inocular tray (May & Houghton, 1964). Selected marker media were then serially inoculated with these suspensions.

Antibiotic marker media. These media consisted of medium NA-1 containing antibiotics at the following concentrations: 5 i.u. penicillin G/ml.; or 10 μg. tetracycline/ml.; or 100 or 1000 μg. streptomycin/ml.; or 2 or 20 μg. chloramphenicol/ml.; or 0·3 μg. erythromycin/ml.; or 1000 μg. sulphadiazine/ml. Selection of the concentrations was based on the known susceptibility spectrum of *Staphylococcus aureus* E 169; the aim was to detect significant increases or decreases in susceptibility.

Fermentation marker media. Media for the detection of acid production from carbohydrates consisted of medium NA-2 (medium NA-1 with the glucose and phosphates omitted), into each 100 ml. of the molten cooled medium was mixed 5 ml. of an indicator solution (containing bromcresol purple 0·1 g., 0·05 N-NaOH 3·7 ml., distilled water to 250 ml.) and 5 ml. of a 20% solution of the appropriate carbohydrate. Indicator and carbohydrate solutions had been previously autoclaved separately at 115° for 10 min. The carbohydrates used were lactose, glucose, mannitol, maltose and sucrose.

Auxotrophic marker medium. A medium designed to detect any increased nutritional requirements consisted of 1% 'Oxoid' Ionagar containing three solutions of salts (A, B and C; compositions described below) and the following nutrients in the stated amounts/ml.: glucose, 2 mg.; glutamic acid, 1 mg. (neutralized with N-NaOH); histidine, glycine, arginine, proline and pantothenic acid, 10 μg.; thiamine and nicotinic acid, 1 μg. Solutions A, B and C each consisted of 100 ml. of distilled water in which were dissolved the following compounds: (A) K_2HPO_4, 70 g.; KH_2PO_4, 30 g.; (B) $MgSO_4.7H_2O$, 1 g.; NaCl and $MnSO_4.4H_2O$, 0·2 g.; (C) $(NH_4)_2$-

SO_4 $FeSO_4.H_2O$ and citric acid, 0·15 g. All nutrient and salt solutions were autoclaved separately at 115° for 10 min. and added to the molten cooled agar solution. Solutions A, B and C were incorporated at the respective rates of 0·5 ml., 0·5 ml., and 0·05 ml./100 ml. medium.

Haemolysin marker medium. Into 100 ml. of molten cooled medium NA-1 were mixed 7 ml. of a sterile suspension of sheep red cells, prepared by washing citrated blood three times with saline and resuspending to its original volume. Each plate contained 10 ml. medium; plates were incubated in an atmosphere of 10% CO_2 and 90% O_2. Under these conditions, growth of *Staphylococcus aureus* E169 was surrounded by a zone of complete haemolysis. When intersected by a zone of β-haemolysis, this clear zone was completely suppressed, indicating that *S. aureus* E169 produces α-haemolysin (Elek & Levy, 1954).

Phosphatase marker medium. Phosphatase was detected by the procedure of Barber & Kuper (1951), except that medium NA-2 was used as the base agar.

Coagulase marker medium. Into 80 ml. of molten NA-1 medium at 50° were mixed 20 ml. of fresh sterile human plasma, and soya-bean trypsin inhibitor (L. Light and Co. Ltd., Colnbrook, England) to a final concentration of 5 μg./ml. (Klemperer & Haughton, 1957). Correctly prepared plates were quite transparent when set.

Staphylokinase marker medium. Into 80 ml. of molten NA-1 medium at 50° were mixed 20 ml. of fresh sterile human plasma; the mixture was then heated at 56° for 30 min. and the plates poured. Correctly prepared plates showed an even haziness. On this medium, colonies of *Staphylococcus aureus* E169 were surrounded by zones of punctate lysis, i.e. Muller's phenomenon, indicating the production of staphylokinase (Hutchison, 1962).

Penicillinase marker medium. Medium and detection procedure were as described by Foley & Perret (1962).

Phage typing. Phage typing was carried out by the procedures laid down by Blair & Williams (1961).

Lysogenicity. The presence of free phage in filtrates of actively growing cultures (population density equivalent to about 0·1 mg. dry wt./ml.) was determined by the agar-layer method of Gratia (Adams, 1959).

RESULTS

Growth and loss of antibiotic resistance in nutrient broth

Although *Staphylococcus aureus* E169 grew in tubes of nutrient broth (medium NB-1) immersed in a water bath regulated at about 44°, little or no growth was obtained when the temperature of the culture, instead of that of the bath, was accurately controlled at 44°. Figure 1 shows that, following transfer from 37° to 44°, the first two doublings were achieved in about 22 min. each; this corresponds to a shortening of the doubling time at 37° by 4 min. Thereafter, the growth rate rapidly decreased; the culture failed to complete the third doubling, and began to lyse between 105 and 180 min. after the temperature shift. The inability of *S. aureus* E169 to grow at 44° necessitated the lowering of the growth-temperature to 43°.

The nature of the growth and loss phenomena when *Staphylococcus aureus* E169 was grown in medium NB-1 at 43° is shown in Figs. 2 and 3. Following transfer to the higher temperature, the first two doublings of the culture occupied about 21 min.

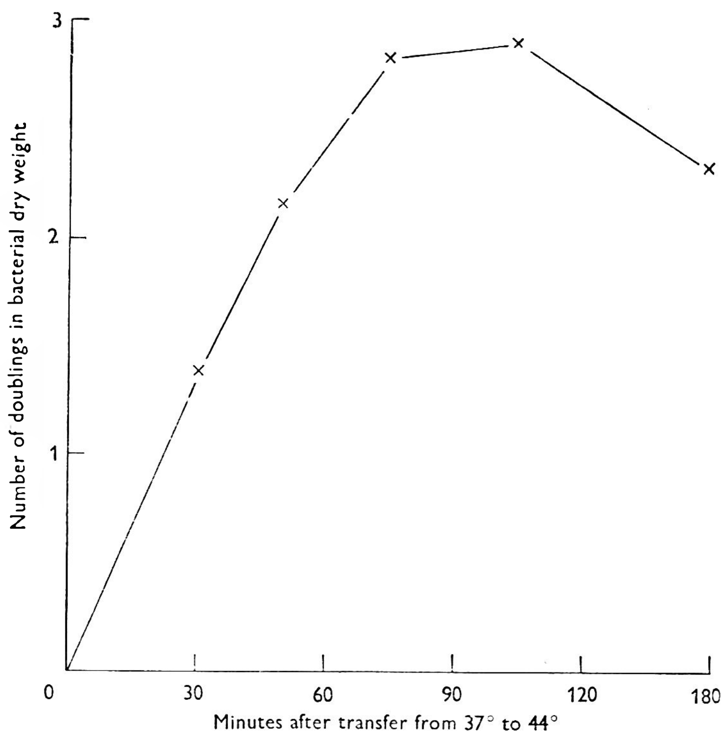


Fig. 1. Cumulative growth curve of Staphylococcus aureus E 169 in medium NB-1 following transfer from 37° to 44°.

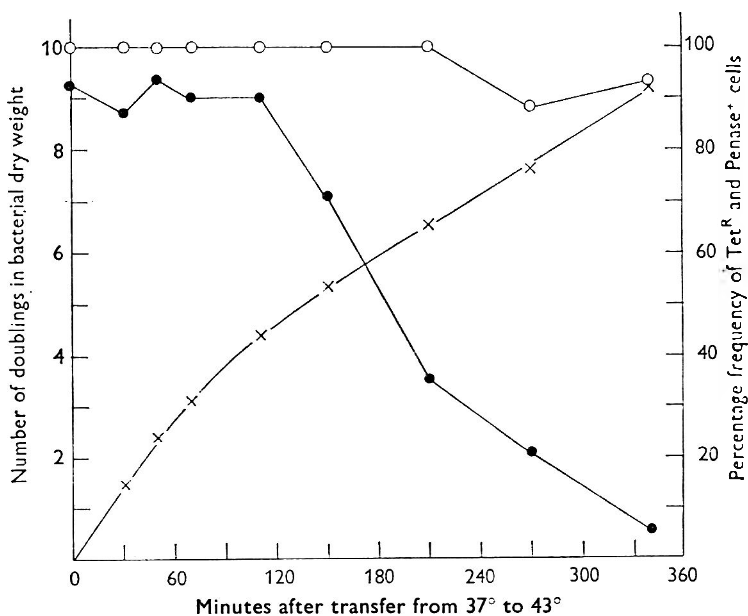


Fig. 2. Cumulative growth curve (×—×) and frequency of Tet^R cocci (●—●) and of Penase$^+$ cocci (○—○) of Staphylococcus aureus E 169 in medium NB-1 following transfer from 37° to 43°.

each. Between the second and fifth doublings, the growth rate slowly declined producing an inflexion in the growth curve; thereafter the doubling time of the culture remained constant at about 50 min. Up to about the fifth doubling, the proportion of Tet^S and $Penase^-$ cocci remained at their 37° values, i.e. about 10 and 1 %, respectively. Thereafter, the proportion of Tet^S cocci increased so rapidly that the Tet^R cocci were 'diluted-out' with a 'halving-time' almost equal to the doubling-

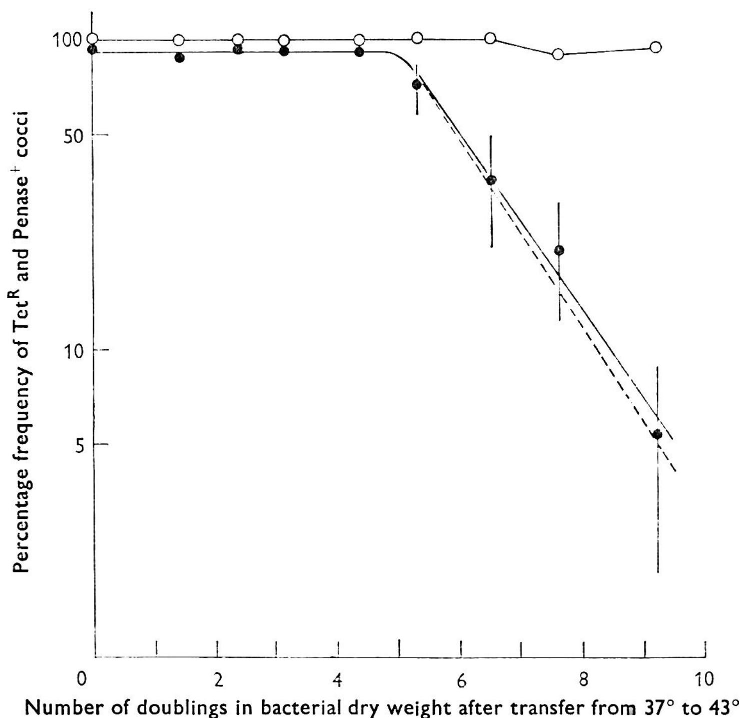


Fig. 3. Relationship between the number of doublings in bacterial dry weight completed by Staphylococcus aureus E 169 in medium NB-1 following transfer from 37° to 43°, and the frequency of Tet^R cocci (●—●) and of $Penase^+$ cocci (○—○). The broken line corresponds to what would be the expected frequency of hypothetical 'passive' particles, i.e. ones that are neither replicated nor destroyed during growth. The vertical lines indicate 95 % confidence limits.

time of the culture (the number of halvings in percentage frequency of Tet^R cocci/doubling of bacterial mass was 0·94). Starting a little later, $Penase^+$ cocci were also 'diluted-out', but at a much slower rate than the Tet^R cocci.

When approximately 10^{11} organisms of Tet^S variants and 10^9 organisms of $Penase^-$ variants, isolated at both 37° and 43°, were plated on to medium NA-1 containing either tetracycline 3 μg./ml. or penicillin G 1 i.u./ml., no spontaneous revertant colonies were observed. The Tet^S and $Penase^-$ variants were therefore extremely stable.

Growth and loss of antibiotic resistance in meat infusion broth

At this stage, the initial batch of 'Oxoid' Nutrient Broth became nearly exhausted, and it was found that the new batch was useless for our purposes. At 43° media

prepared from the new batch gave such extremely granular cultures that extinction measurements were impossible. Tests of other readily available media revealed that our meat infusion broth (MIB) gave fully dispersed cultures even at 44°, the temperature which was originally used by Fairbrother *et al.* (1954). MIB and a growth temperature of 44° were therefore adopted for all subsequent experiments; typical results are shown in Fig. 4. The decline in the proportion of Tet^R and $Penase^+$

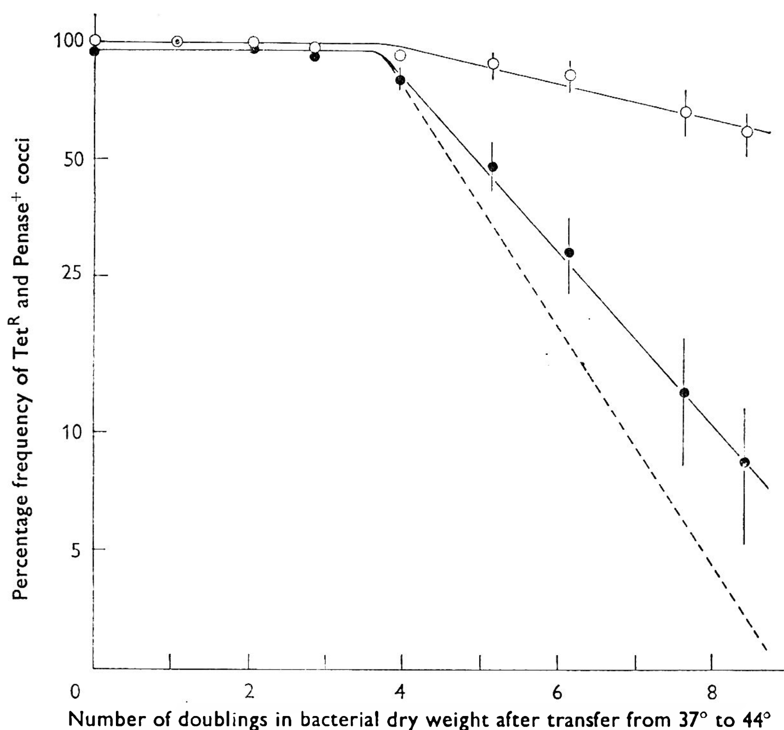


Fig. 4. Relationship between the number of doublings in bacterial dry weight completed by Staphylococcus aureus E169 in MIB following transfer from 37° to 44°, and the frequency of Tet^R cocci (●—●) and of $Penase^+$ cocci (○—○). The broken line corresponds to what would be the expected frequency of hypothetical 'passive' particles, i.e. ones that are neither replicated nor destroyed during growth. The vertical lines indicate 95 % confidence limits.

cocci started between the third and fourth doublings, which was earlier than in medium NB-1 at 43°, and by the fifth doubling the proportion of Tet^R cocci had fallen to approximately 50 %. However, the 'dilution-out', at least for Tet^R cocci, proceeded at a slower rate than in medium NB-1. Thus, between approximately the fourth and eighth doublings, the number of halvings in percentage frequency of Tet^R cocci/doubling of bacterial mass, was 0·73. Examination of bacteria stained to reveal cell walls showed that the mean number of cells per clump (excluding the tail of the frequency distribution above the ninth decile) was approximately 2, with median and mode of 2, prior to transfer from 37° to 44°; and was approximately 4, with median and mode of 4, after 5 or more doublings at 44°.

Since one possible explanation of the observed population changes was that the parental type virtually stopped reproducing (while remaining viable) and was

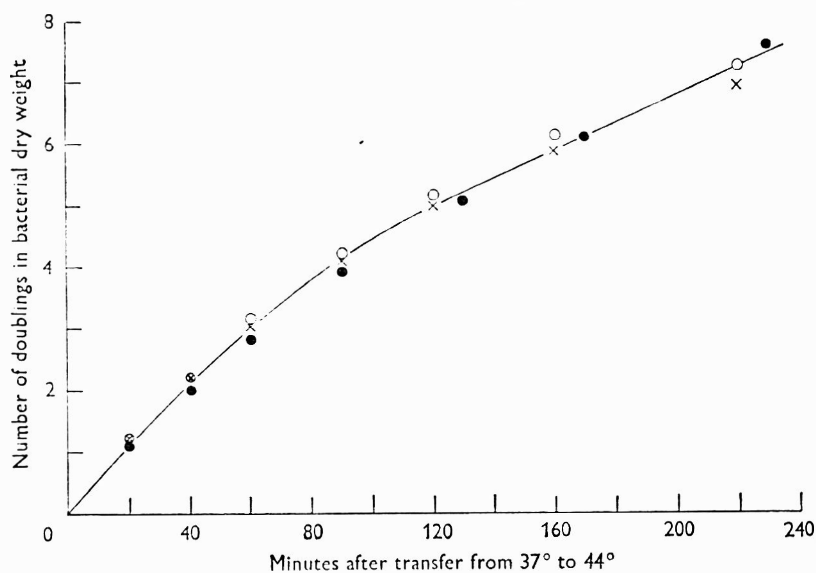


Fig. 5. Cumulative growth curves in MIB following transfer from 37° to 44° of Staphylococcus aureus E 169 (●—●), and of Tet^S variants isolated from cultures grown at either 37° (○—○) or at 44° (× — ×).

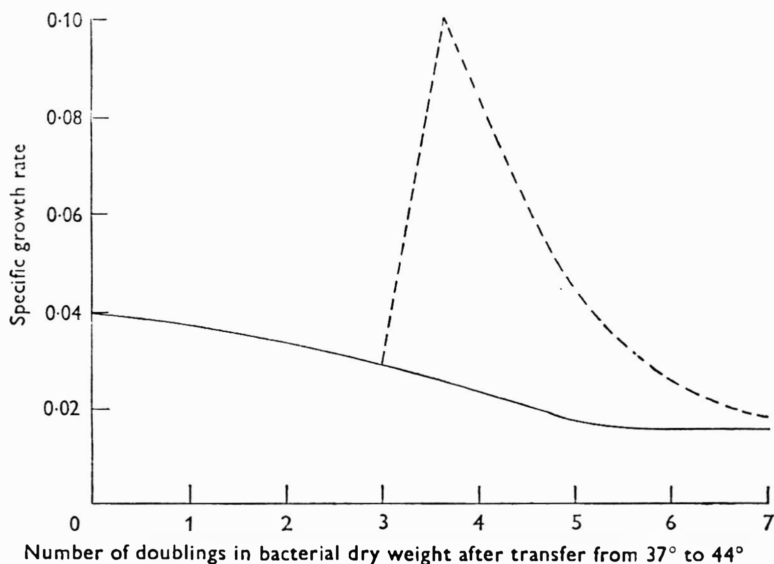


Fig. 6. The relationship between specific growth rate and the number of doublings in bacterial dry weight completed by Staphylococcus aureus E 169 in MIB following transfer from 37° to 44°. The unbroken line shows the specific growth rate of the whole culture taken from Fig. 5. The broken line indicates the specific rate at which the Tet^S variants would need to grow in order to account for the observed decrease in percentage frequency of Tet^R cocci shown in Fig. 4.

overgrown by the variants, the growth curves at 44° of Tet^S variants isolated at both 37° and 44° were compared with that of the original *Staphylococcus aureus* E 169; Fig. 5 shows that the growth curves of the three strains were in fact almost identical. Fig. 6 shows the specific growth rates of the three strains (calculated from the fitted curve in Fig. 5), together with the specific growth rate which would be needed if the Tet^S variants were to overgrow the Tet^R cocci at a rate which would explain the observed proportional diminution of the latter revealed in Fig. 4. It seems clear from Fig. 6 that the observed changes in the Tet^R:Tet^S ratio could not possibly be caused by differences in the growth rates of the two types.

Table 1. *Effect of growth in MIB at 44° upon various marker properties of Staphylococcus aureus* E169

Property	Percentage frequency of cocci positive after *n* doublings in bacterial dry weight at 44°	
	n = 0	*n* = 9·3
Growth on		
Penicillin (5 i.u./ml.)	100	58
Tetracycline (10 μg./ml.)	98	1·3
Streptomycin (100 μg./ml.)	100	100
Streptomycin (1000 μg./ml.)	0	0
Chloramphenicol (2 μg./ml.)	100	100
Chloramphenicol (20 μg./ml.)	0	0
Erythromycin (0·3 μg./ml.)	0	0
Sulphadiazine (1000 μg./ml.)	100	100
Auxotrophic medium	100	100
Production of		
Penicillinase	100	58
Coagulase, phosphatase, staphylokinase, haemolysin	100	100
Fermentation of		
Lactose, glucose, mannitol, maltose, sucrose	100	100

Effect of growth at elevated temperature on other properties. To discover whether the 'instability' of tetracycline resistance and penicillinase-forming ability of *Staphylococcus aureus* E169 was shared by other properties, a doubling-dilution growth-run, lasting for about nine doublings in MIB at 44°, was accompanied by replication of colonies on a wide range of 'marker' media. Table 1 shows the percentage composition of the bacterial population with respect to the various properties at the start and finish of the experiment. It can be seen that tetracycline resistance, production of penicillinase and penicillin resistance were the only properties which showed any change within the range of sensitivity allowed by the limited number of colonies tested. All marker media gave unequivocal results, except the lactose fermentation medium. *S. aureus* E169 is a slow or weak lactose fermenter; nevertheless, it did appear that some of the colonies from the final sample had produced even less acid than usual. But no difference between these colonies and normal *S. aureus* E169 was detected when a comparison was made in the standard lactose-peptone water. The culture was therefore recorded as showing no change with respect to lactose fermentation.

ห้องสมุด กรมวิทยาศาสตร์

Staphylococcus aureus E169 is lysogenic, releasing one or more phages active on *S. aureus* NCTC 9789 (the propagating strain for staphylococcal phage 80). However, loss of either tetracycline resistance or penicillinase-forming ability was not associated with loss of prophage since none of three different heat-induced variants (Tet^R $Penase^-$; Tet^S $Penase^+$; and Tet^S $Penase^-$) showed any alteration in phage type and all were immune to phage released by the parental type.

Effect of acriflavine treatment and ultraviolet irradiation upon the frequency of Tet^S and $Penase^-$ variants. Following growth in MIB containing 0·1 mg. acriflavine/ml., of 200 colonies tested 6 % were Tet^S and 2 % were $Penase^-$; similarly, when about 5×10^8 cocci were subjected to ultraviolet irradiation, of the approximately 200 colonies surviving, 5 % were Tet^S and 1 % were $Penase^-$. Therefore neither of these two agents significantly affects the frequency of Tet^S or $Penase^-$ variants in populations of *Staphylococcus aureus* E169.

Table 2. *Frequency of tetracycline-sensitive variants of Staphylococcus aureus* E169 *in colonies and their serial MIB subcultures grown at* 37°

Colony no.	Percentage of Tet^S variants*: In suspension of whole colony picked from MIA	In serial MIB subcultures of colony suspension: 1st	2nd	3rd
1	7	4	6	5
2	4	6	4	3
3	3	4	7	9
4	22	22	19	26
5	< 1	7	9	14

* One hundred colonies screened for each estimate.

Frequency of Tet^S variants in cultures grown at 37°. The frequency of Tet^S variants in five colonies of *Staphylococcus aureus* E169 picked from MIA and in three serial MIB subcultures of the colony suspensions are shown in Table 2. The frequency of Tet^S variants attained during growth of four of the five colonies did not significantly alter during serial subculture. In the fifth colony, the frequency appeared to increase during the first subculture in MIB, but then stabilized at about 10 %; the level around which the frequency in the four other series tended to cluster. These results suggested that the equilibrium frequency of Tet^S variants in cultures of *S. aureus* E169 grown at 37° was about 10 %. Nevertheless, when logarithmically growing cultures of *S. aureus* E169 and one of its Tet^S variants isolated at 37° were mixed to give a culture containing approximately 90 % Tet^S cocci, there was no significant alteration in the frequency of Tet^S cocci after about 14 population doublings of the artificially mixed culture at 37°.

DISCUSSION

The occurrence of a rather high number of Tet^S and $Penase^-$ colonies when the nominally Tet^R $Penase^+$ *Staphylococcus aureus* E169 was plated-out might be regarded as evidence that, in spite of its mode of isolation, it was a mixed culture. Nevertheless, we are persuaded that the explanation is that the staphylococcus is

inherently unstable with respect to tetracycline resistance and production of penicillinase.

The different rates of loss of tetracycline resistance and penicillinase-forming ability by *Staphylococcus aureus* E169 during growth at elevated temperature, with the consequent occurrence in 'terminal' populations of both Tet^R $Penase^-$ and Tet^S $Penase^+$ in addition to Tet^S $Penase^-$ variants, shows that these two properties are lost independently. Furthermore, these losses at elevated temperature are clearly not environmentally induced phenotypic changes: the Tet^S and $Penase^-$ variants are quite stable during repeated subculture at 37°, and the rates of reversion are less than $1/10^9$ cocci; indeed, no revertants have yet been observed.

Of the possible hypotheses which might be invoked to explain the phenomenon, several are highly improbable in the light of current evidence from other sources. For instance, heritable properties of staphylococci dependent on the presence of prophage have been reported by a number of workers (Blair & Carr, 1961; Wahl & Fouace, 1961; de Waart, Winkler & Grootsen, 1962). However, since the Tet^S and $Penase^-$ variants are immune to phage released by the parental strain, it does not seem possible that the observed alterations are associated with the loss of prophage. Neither does it appear possible that Tet^S variants present in the culture before exposure to elevated temperature selectively overgrow the Tet^R cocci. Such an overgrowth would require a doubling-time for the Tet^S cocci of approximately 6·5 min. between the third and fourth doublings in MIB at 44°, whereas the doubling times of both types were then about 27 min. (Fig. 5 and 6).

It is also unlikely that the changes are heat-induced mutations of the type described by Zamenhof & Greer (1958). The speed with which the variants accumulate in the population in the absence of any demonstrable growth advantage would require an extraordinarily high mutation rate, particularly in the case of the Tet^R to Tet^S variation. The stability of the numerous other 'marker' properties at 44° also weighs somewhat against a mutational interpretation, although one could suppose that the Tet^R and $Penase^+$ loci were situated at heat-sensitive 'hot-spots' on the genome. But it would be necessary further to assume that such 'hot-spots' were unaffected by ultraviolet irradiation.

A more attractive hypothesis is that tetracycline resistance and penicillinase-forming ability are determined by autonomously-replicating cytoplasmic particles whose doubling rates are equal to the cell doubling rate at 37°, but whose replication is more or less suppressed at higher temperatures; under these conditions the particles would be diluted out until there was only one per cell, thereafter being inherited unilinearly in a 'one or none' manner (see Stocker, 1956). Thus with *Staphylococcus aureus* E169 growing in MIB, since the number of cocci per clump was approximately 2 at the time of transfer from 37° to 44° and 4 at the fifth population doubling at 44°, when about half of the clumps in the population were Tet^R, then at the time of transfer each cell would have contained about 4 Tet^R-conferring particles. However, since there was no significant change in the state of aggregation of cocci beyond the fifth population doubling at 44°, the rate of disappearance of Tet^R cocci during the same period suggests that the replication of the particles was not completely suppressed at this temperature and the estimate of four particles per cell is probably too high. The six doublings required at 43° in medium NB-1 for half of the population to be Tet^R was probably due to the clumps of cocci being larger in this medium;

indeed, medium NB-1 made from a second batch of 'Oxoid' Nutrient Broth granules even gave visible clumping of cultures grown at 37°. This distinction between MIB and medium NB-1, together with the inability of the latter to sustain the growth of *S. aureus* E169 at 44°, was presumably due to the differences in composition of the two media.

Recently, Jacob, Brenner & Cuzin (1963) have described mutants of the sex factor in *Escherichia coli*; in the cytoplasmic state the mutant episomes were eliminated by growing the bacteria at 42°, but were temperature resistant when integrated with the chromosome. As yet, we have no evidence to suggest that particles conferring tetracycline resistance and penicillinase-forming ability on *Staphylococcus aureus* E169 are episomes (Jacob & Wollman, 1958) and consequently we have called them plasmids (Lederberg, 1952).

The insensitivity of the plasmids to acriflavine conflicts with previously reported properties of autonomously-replicating cytoplasmic particles conferring antibiotic resistance in *Escherichia coli* (Watanabe & Fukasawa, 1961) and in *Staphylococcus aureus* (Mitsuhashi, Morimura, Kono & Oshima, 1963). But Novick (1963) also concluded, from transductional and other genetic evidence, that penicillinase formation in *S. aureus* was controlled by an acridine-dye resistant plasmid.

The value of about 10% Tet^S variants in the predominantly Tet^R cultures derived from single colonies of *Staphylococcus aureus* E169 suggested that this represented the equilibrium of the two types when growing at 37°, the random failure of a daughter cell to inherit any Tet^R-conferring particle from its parent being counterbalanced by re-infection. However, the stability of artificially mixed cultures demonstrated that active reinfection did not occur under our experimental conditions. We are, therefore, at present unable to explain how populations which should seemingly be almost completely tetracycline sensitive actually remain dominantly tetracycline resistant.

We wish to thank Mr L. R. Rowett (Royal Perth Hospital) for performing the phage typing. This work was supported in part by a grant from the National Health and Medical Research Council of Australia.

REFERENCES

ADAMS, M. H. (1959). *Bacteriophages*. New York: Interscience Publishers, Inc.

ANNEAR, D. I. (1962). Recoveries of bacteria after drying on cellulose fibres. A method for the routine preservation of bacteria. *Aust. J. exp. Biol. med. Sci.* **40**, 1.

BARBER, M. (1949). The incidence of penicillin-sensitive variant colonies in penicillinase-producing strains of *Staphylococcus pyogenes*. *J. gen. Microbiol.* **3**, 274.

BARBER, M. & KUPER, S. W. A. (1951). Identification of *Staphylococcus pyogenes* by the phosphatase reaction. *J. Path. Bact.* **63**, 65.

BISSET, K. A. & HALE, C. M. F. (1953). Complex cellular structure in bacteria. *Exp. Cell. Res.* **5**, 449.

BLAIR, J. E. & CARR, M. (1961). Lysogeny in staphylococci. *J. Bact.* **82**, 984.

BLAIR, J. E. & WILLIAMS, R. E. O. (1961). Phage typing of staphylococci. *Bull. Wld Hlth Org.* **24**, 771.

BONDI, A., KORNBLUM, J. & DE SAINT PHALLE, M. (1953). Isolation of penicillin-susceptible mutants from penicillinase-producing strains of *Micrococcus pyogenes*. *Proc. Soc. exp. Biol., N.Y.* **83**, 527.

BOROWSKI, J. (1963). Tendance naturelle des staphylocoques pénicillino-résistants a donner des mutants sensibles à la pénicilline. Relations de cette propriété avec l'origine des souches et leur sensibilité aux bactériophages. *Ann. Inst. Pasteur*, **104**, 535.

ELEK, S. D. & LEVY, E. (1954). The nature of discrepancies between haemolysins in culture filtrates and plate haemolysin patterns of staphylococci. *J. Path. Bact.* **68**, 31.

FAIRBROTHER, R. W., PARKER, L. & EATON, B. R. (1954). The stability of penicillinase-producing strains of *Staphylococcus aureus*. *J. gen. Microbiol.* **10**, 309.

FOLEY, J. M. & PERRET, C. J. (1962). Screening bacterial colonies for penicillinase production. *Nature, Lond.* **195**, 287.

HUTCHISON, J. G. P. (1962). Muller's phenomenon and its relationship to plasminogen activation and to blood constituents producing opacity in an agar gel. *J. Path. Bact.* **84**, 289.

JACOB, F., BRENNER, S. & CUZIN, F. (1963). On the regulation of DNA replication in bacteria. *Cold Spr. Harb. Symp. quant. Biol.* **28**, 329.

JACOB, F. & WOLLMAN, E. L. (1958). Les épisomes, éléments génétiques ajoutés. *C.R. Acad. Sci., Paris*, **247**, 154.

KANTOROWICZ, O. (1951). Shaking apparatus for the aeration of bacterial cultures. *J. gen. Microbiol.* **5**, 276.

KLEMPERER, R. & HAUGHTON, G. (1957). A medium for the rapid recognition of penicillin-resistant coagulase-positive staphylococci. *J. clin. Path.* **10**, 96.

LEDERBERG, J. (1952). Cell genetics and hereditary symbiosis. *Physiol. Rev.* **32**, 403.

Mackie and McCartney's Handbook of Bacteriology (1960). 10th ed. Ed. by R. Cruickshank, p. 190. Edinburgh and London: Livingstone.

MAY, J. W. & HOUGHTON, R. H. (1964). A simple multipoint inoculator. *Lab. Pract.* (in the Press).

MITSUHASHI, S., MORIMURA, M., KONO, K. & OSHIMA, H. (1963). Elimination of drug resistance of *Staphylococcus aureus* by treatment with acriflavine. *J. Bact.* **86**, 162.

NOVICK, R. P. (1963). Analysis by transduction of mutations affecting penicillinase formation in *Staphylococcus aureus*. *J. gen. Microbiol.* **33**, 121.

STOCKER, B. A. D. (1956). Abortive transduction of motility in salmonella; a non-replicated gene transmitted through many generations to a single descendant. *J. gen. Microbiol.* **15**, 575.

DE WAART, J., WINKLER, K. C. & GROOTSEN, C. A. (1962). Lysogenic conversion in Staphylococci. *Nature, Lond.* **195**, 407.

WAHL, R. & FOUACE, J. (1961). Mutations d'une souche de *Staphylococcus aureus* vers la résistance à l'érythromycine avec perte de prophages. *Ann. Inst. Pasteur*, **100**, 553.

WATANABE, T. & FUKASAWA, T. (1961). Episome-mediated transfer of drug resistance in Enterobacteriaceae. II. Elimination of resistance factors with acridine dyes. *J. Bact.* **81**, 679.

ZAMENHOF, S. & GREER, S. (1958). Heat as an agent producing high frequency of mutations and unstable genes in *Escherichia coli*. *Nature, Lond.* **182**, 611.

J. gen. Microbiol. (1964), 37, 171–180
Printed in Great Britain

The Accumulation of *O*-Succinylhomoserine by *Escherichia coli* and *Salmonella typhimurium*

By R. J. ROWBURY

Department of Botany, University College London

(*Received* 21 *April* 1964)

SUMMARY

After growth of *Escherichia coli* strain 7/9, the culture fluid and organisms contained an amino acid which gave a yellow-brown colour with ninhydrin. This compound was shown by comparison with synthetic material to be *O*-succinylhomoserine believed from previous work with this *E. coli* strain to be a methionine precursor. Its accumulation gave proof that it was formed in large amounts by the growing organisms. The formation of *O*-succinylhomoserine occurred equally well in the presence or absence of added homoserine and succinate, but when the succinate was added to growth media, synthesis occurred preferentially from it and the utilization of endogenously-formed succinate was diminished. Without added homoserine the extent of accumulation was such (up to 440 mg. *O*-succinylhomoserine/l.) that homoserine synthesis may have been increased above normal to provide the required quantities (up to 240 mg./l.) of homoserine. Methionine completely suppressed accumulation of *O*-succinylhomoserine, threonine and lysine had no effect. A cystathionine-requiring mutant of *Salmonella typhimurium* also accumulated *O*-succinylhomoserine; the amount (20–30 mg./l.) being only about 5% of that given by *E. coli*, and methionine prevented its accumulation by this organism too. It appears that the methionine synthesis pathway in *S. typhimurium* is very similar to that in *E. coli* and is subject to end-product control.

INTRODUCTION

For many biosynthetic pathways the occurrence of compounds as intermediates has been established or confirmed by their isolation from organisms or their cultures. For example, the role of homoserine as a methionine precursor in Neurospora was shown by its accumulation in a methionine-requiring strain (Fling & Horowitz, 1951) and the isolation of cystathionine from another methionine-requiring strain of the same organism (Horowitz, 1947) suggested that cystathionine was an intermediate. The accumulation of methionine precursors by *Escherichia coli* and *Salmonella typhimurium* has not been reported previously; Harrold (1962) observed cystathionine accumulation by a mutant of *Aerobacter aerogenes*. During studies with a methionine-requiring strain of *E. coli* it was observed that the organisms accumulated what appeared to be *O*-succinylhomoserine. The functioning of homoserine and succinate in methionine synthesis had been suggested by studies with auxotrophic mutants of *E. coli* (Davis, 1955; Davis *et al.* 1959) and *O*-succinylhomoserine had been shown as an intermediate of cystathionine synthesis by experiments with cell-free extracts (Rowbury, 1962*b*, *c*; Rowbury & Woods, 1964). This accumulation was studied to obtain conclusive evidence that *O*-succinylhomoserine is a normal intermediate of methionine synthesis in *E. coli*.

The growth characteristics of methionineless mutants of *Salmonella typhimurium* suggest that the synthesis pathway is similar to that in *Escherichia coli* (Smith, 1961), with homoserine, cystathionine and homocysteine as intermediates. Cystathionine-requiring mutants of this organism were therefore screened to ascertain whether accumulation of *O*-succinylhomoserine occurred.

METHODS

Organisms

The organisms employed were all auxotrophic strains of *Escherichia coli* and *Salmonella typhimurium* and were maintained on slopes of Oxoid nutrient agar, subcultured monthly and stored at 4° after incubation for 18 hr. The strains used were *E. coli* strains 122/33, 7/9, 2/2, 26/18 and B184 and *S. typhimurium* strains A15 and B23. Strains 122/33, 7/9, 2/2, A15 and B23 all grew on minimal medium supplemented with methionine or homocysteine or cystathionine. Strain 26/18 required homocysteine or methionine for growth while strain B184 required homoserine or methionine plus threonine for growth. Strains 26/18, 7/9 and 2/2 were isolated from strain 518, strain 122/33 was isolated from strain W by Dr B. D. Davis and strain B184 which derived from strain B was provided by Dr J. Gots. The two strains of *S. typhimurium* were obtained from Dr D. A. Smith.

Growth of organisms and preparation of enzymic extracts

The medium used was the glucose, lactate and salts medium (GL) of Guest, Helleiner, Cross & Woods (1960). The medium was supplemented with DL-homoserine (0·3 mM) for strain B184 and with DL-homocysteine (0·25 mM) for the other strains. Organisms were harvested and enzymic extracts prepared as described previously (Rowbury & Woods, 1961). The heated extract of organisms from strain 7/9 was prepared by incubating bacteria (equiv. 50 mg. dry-wt./ml.) in water at 100° for 10 min. The supernatant fluid after centrifugation (10,000*g* for 30 min.) was used as the heated extract.

The assay of O-succinylhomoserine. The cystathionine formed from the compound on incubation in solution *A* was measured. Solution *A* contained (in phosphate buffer pH 7·5, 133 mM) cysteine (10 μmole), ATP (10 μmole), glucose (20 μmole) and extract from strain 2/2 (5 mg. protein). The cystathionine formed was assayed microbiologically with *Escherichia coli* strain 122/33 as described previously (Rowbury & Woods, 1964). For comparison purposes samples of synthetic *O*-succinylhomoserine and the compound formed by enzymic extracts were used. The synthetic compound was formed from homoserine and succinic anhydride as described by Rowbury & Woods (1964). The biologically formed *O*-succinylhomoserine was made by incubation of DL-homoserine (15 μmole), sodium succinate (150 μmole), ATP (15 μmole), glucose (20 μmole) and coenzyme A (0·02 μmole) with extract from strain 7/9 (5 mg. protein) in phosphate buffer (133 mM, pH 7·5).

Chromatography

Paper chromatograms were run in *n*-butanol + propionic acid + water (47 + 22 + 31) descending for 16 hr. Amino acids were detected by spraying with ninhydrin (0·2 % in *n*-butanol saturated with water). In certain experiments the chromatograms were

scanned for isotope directly by using the Geiger–Müller tube of a Ratemeter Unit (type 1355B, Fleming Radio Developments Ltd.).

The *O*-succinyl derivative of homoserine was also chromatographed on columns of Dowex-1 resin. Samples were applied to 18 × 0·9 cm. columns of resin (× 8, 200–400 mesh, acetate form) in aqueous solution. Elution was with water (15 ml.) then with 0·2 N-acetic acid. Biological activity of the fractions was assayed with strain 2/2 extract after evaporation to dryness to remove acetic acid. Amino nitrogen in the eluent was assayed with a modified ninhydrin reagent (Moore & Stein, 1954). In certain cases the radioactivity of the eluent was assessed by spotting samples on uniform ground glass discs and counting at infinite thinness with a Scaler 1700 (Isotope Developments Ltd.) for 1000 sec. Corrections were made for background activity.

Chemicals

The sources of most of the special chemicals used were as described previously (Rowbury & Woods, 1964). $^{14}C_{2,3}$-succinate was obtained from the Radiochemical Centre, Amersham.

RESULTS

The isolation of O-succinylhomoserine from Escherichia coli strain 7/9

Escherichia coli strain 7/9 (a strain unable to form cystathionine) was previously used for studies on the synthesis of *O*-succinylhomoserine by enzymic extracts (Rowbury, 1962*a*, *b*; Rowbury & Woods, 1964) as it lacks the ability to condense cysteine with *O*-succinylhomoserine. In the present work the organisms were grown with limiting amounts of homocysteine (0·25 mM, DL) as methionine was known to prevent synthesis of *O*-succinylhomoserine (Rowbury, 1962*a*) and, after harvesting, the growth medium was concentrated and a heated extract prepared from the organisms. The medium and heated extract both showed a ninhydrin positive spot at R_F 0·35 when chromatographed on paper in *n*-butanol + propionic acid + water. The compound gave a yellow brown colour (which later turned purple) on heating with ninhydrin. Both synthetic and biologically formed *O*-succinylhomoserine ran at R_F 0·35 in this solvent and the yellow brown colour with ninhydrin was characteristic of this compound.

The substance present in the medium and organisms gave rise to cystathionine when incubated with ultrasonic extract from strain 2/2 or strain 26/18. As with synthetic *O*-succinylhomoserine and that formed by enzymic extracts, cysteine was required for cystathionine formation, but homoserine and succinate were not (Table 1). Although extracts from strain 2/2 made cystathionine from the accumulated compound, the growth of strain 2/2 was not stimulated by it (as a replacement for cystathionine) and hence cross-feeding did not occur between strains 7/9 and 2/2. Thus a sample of the accumulated compound (1 μmole/ml.) had less that 3 % of the growth promoting activity of cystathionine for strain 2/2.

Very large amounts of the cystathionine precursor were present in the growth medium of strain 7/9 but lesser amounts in the organisms (Table 2). During the early stages of this work, homoserine and succinate were added to growth media but this was later shown to be unnecessary (Table 2) as these compounds did not increase the accumulation. Under optimal conditions up to 2000 μmole of the precursor were formed per litre of medium.

Other strains of *Escherichia coli* were tested to ascertain whether the cystathionine precursor accumulated. Three strains were tested, one (strain 2/2) unable to form cystathionine, a second which is unable to carry out a step before cystathionine formation (strain B184, a homoserine requirer, lacking homoserine dehydrogenase) and a third strain lacking the cystathionase enzyme (strain 26/18). None of these strains accumulated the precursor.

Table 1. *The synthesis of cystathionine from the accumulated compound and from synthetic O-succinyl homoserine*

Organisms (Escherichia coli strain 7/9) were harvested from medium GL+homocysteine, the culture fluid concentrated by evaporation *in vacuo*, and samples incubated in 3 ml. of solution A at 37° with the stated omissions and additions. The *O*-succinyl-homoserine was a synthetic sample purified on Dowex-1 resin.

	Cystathionine formed (μmmole/mg. protein)	
Additions	From the accumulated compound	From *O*-succinyl homoserine (2 mM)
None	286	240
None (cysteine omitted)	8	0
Homoserine (5 mM)	280	230
Succinate (50 mM)	290	220

Table 2. *The extent of the accumulation of the cystathionine precursor in Escherichia coli strain 7/9*

Organisms were grown on medium GL+homocysteine (0·3 mM) with the stated additions for 18 hr at 37°. In all cases the medium was concentrated *in vacuo*, and in one case a heated extract of the organisms was prepared. The precursor was assayed by cystathionine formation in solution A.

	Cystathionine precursor (μmole/l. culture)	
Additions	In organisms	In medium
None	—	1640
Homoserine (2 mM) + succinate (20 mM)	60	1400
Homoserine (2 mM)	—	1480
Succinate (20 mM)	—	1550

The purification and properties of the accumulated compound

Homoserine and other neutral amino acids can be readily removed from samples of *O*-succinylhomoserine by chromatography on Dowex-1 resin (Rowbury & Woods, 1964) as *O*-succinylhomoserine (due to its slight acidity) is retained by the resin when a neutral solution is applied, but is eluted with 0·2 N acetic acid. The cystathionine precursor accumulated by strain 7/9 was also retained by the resin and on addition of 0·2 N acetic acid was eluted in the same position as *O*-succinylhomoserine (Fig. 1).

A purified sample of the accumulated compound was very unstable to mild alkali but more stable in mild acid solution (Fig. 2); extreme instability to alkali was a characteristic of *O*-succinylhomoserine (Rowbury, 1962*b*; Rowbury & Woods, 1964), and the two compounds behaved very similarly over the range pH 2–11 (Fig. 2).

Although the precursor was stable to treatment with mild acid, more vigorous conditions (N-HCl for 2 hr at 100°) destroyed its biological activity and paper chromatography showed the presence of homoserine in the hydrolysate.

The presence of succinate in the cystathionine precursor

When studying *O*-succinylhomoserine formation by enzymic extracts, the incorporation of succinate was shown by using ^{14}C-succinate (Rowbury, 1962*b*). To demonstrate the presence of a succinate residue in the accumulated compound,

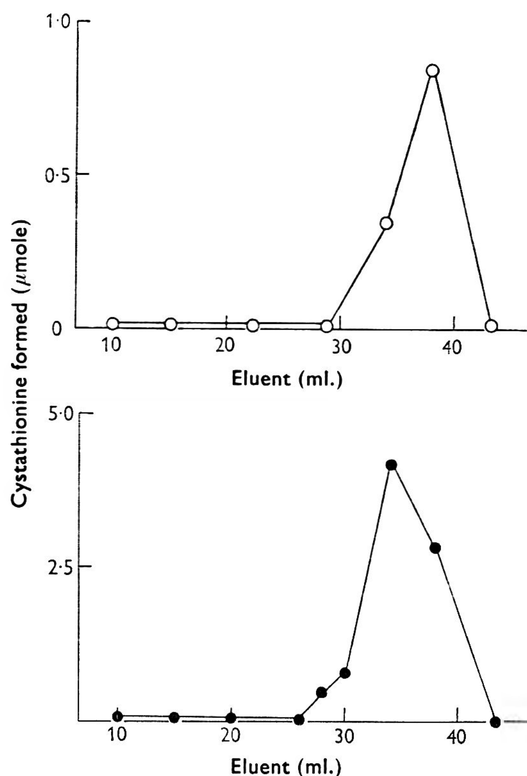


Fig. 1. Comparison of the behaviour of the accumulated cystathionine precursor and synthetic *O*-succinylhomoserine on Dowex-1 resin. Samples of the precursor and of synthetic compound were applied to columns of Dowex-1 resin and eluted as described in Methods. Biological activity was assayed by incubation in solution A. ●, synthetic *O*-succinyl homoserine; ○, cystathionine precursor.

organisms were grown on medium GL with limiting homocysteine and with ^{14}C-succinate added. When the concentrated growth medium was chromatographed on Dowex-1 resin, two peaks of isotope were observed (Fig. 3). The first peak probably represented incorporation into neutral amino acids; the second peak was due to incorporation of succinate into the precursor as it coincided with the peak of biological activity (i.e cystathionine formation when incubated with strain 2/2 extract in solution *A*). On paper chromatography of a sample from this second peak, all the isotope ran at R_F 0·35 (in *n*-butanol + propionic acid + water) coinciding with the spot of ninhydrin-positive material.

An appreciable amount of the added ^{14}C-succinate was incorporated into the precursor even when unlabelled succinate was added to the growth medium (Table 3). When 10 mM succinate was added, 10 % of the isotope was incorporated into the precursor and with 25 mM succinate present, 5 % was incorporated. When the amount of the precursor (in terms of biological activity) was compared to the extent of isotope incorporation it was apparent that most of the accumulated compound had arisen from added succinate rather than from endogenously formed succinate

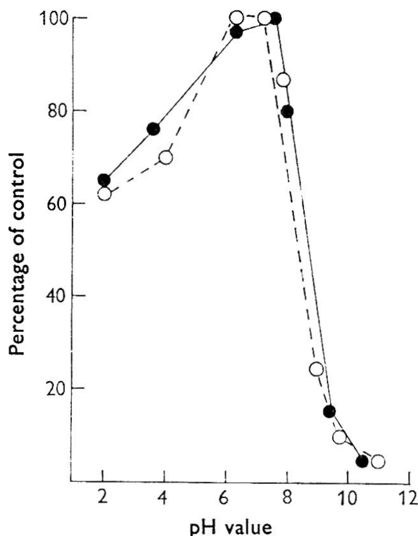


Fig. 2

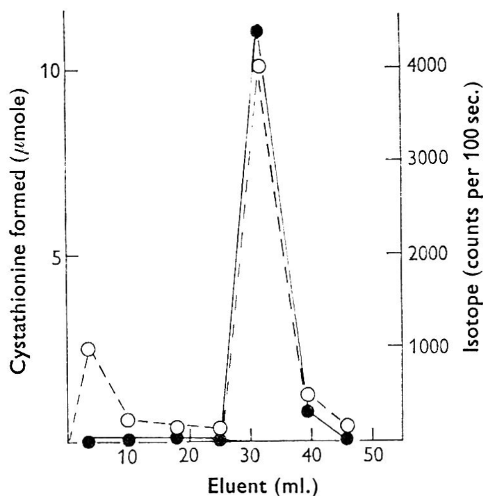


Fig. 3

Fig. 2. The effect of mild acid and alkali on accumulated precursor. Samples of the accumulated compound and synthetic *O*-succinylhomoserine were incubated at the stated pH value for 5 min. at 37°, then readjusted to pH 7·5 and incubated in solution A for 4 hr. Cystathionine formed was assayed with Escherichia coli strain 122/33. ●, synthetic *O*-succinylhomoserine; ○, the accumulated cystathionine precursor.

Fig. 3. The presence of a succinate residue in the accumulated cystathionine precursor. Organisms of Escherichia coli strain 7/9 were grown on medium GL with added $^{14}C_{2,3}$-succinate and the medium chromatographed on Dowex-1 resin as described in Methods. Biological activity of the fractions was measured in solution *A* and radioactivity assessed as described in Methods. ●, biological activity; ○, radioactivity.

Table 3. *The incorporation of exogenous succinate into the cystathionine precursor*

Organisms (Escherichia coli strain 7/9) were grown on medium GL + homocysteine with the stated concentration of succinate as well as ^{14}C-succinate (5 μc. total). The culture fluid (10 ml. total) was concentrated *in vacuo* and applied to a column of Dowex-1 resin. Samples of the eluate were assayed for isotope as described in Methods and for biological activity by incubation in solution A.

Succinate in medium (mM)	Cystathionine precursor formed		
	A. Total (μmole)	From ^{14}C-succinate (μmole)	% from ^{14}C-succinate
10	11·2	9·2	82
25	12·0	11·0	92

(Table 3). When 25 mM succinate was present 92 % of the precursor had arisen from exogenous succinate and only 8 % from endogenous sources.

The effect of methionine on the accumulation of the cystathionine precursor

Accumulations generally occur only after the growth of cultures has ceased and when any added metabolite has been exhausted (Gots, 1957). For this reason the experiments described above were with organisms grown with limited quantities of homocysteine. When DL-methionine (3·3 mM) was added to the medium, it was observed by paper chromatography that the cystathionine precursor was not accumulated; this was confirmed by chromatography on Dowex-1 resin columns. The precursor was separated from methionine (which would interfere with the bioassay of cystathionine) and assayed for cystathionine synthesis in solution *A* or with ninhydrin (Fig. 4). Methionine reduced the accumulation of the precursor to less than 2 % although lysine and threonine (which have partly common biosynthetic pathways with methionine) did not prevent the accumulation when added to media (Table 4).

Table 4. *The effect of methionine, threonine and lysine on the accumulation of the cystathionine precursor*

Organisms (Escherichia coli strain 7/9) were grown on medium GL + homocysteine with the stated additions for 18 hr at 37°, and the culture fluid (50 ml.) concentrated *in vacuo*. Where methionine had been added, the medium was chromatographed on Dowex-1 resin and the appropriate fractions assayed in solution A for the cystathionine precursor. The other samples were assayed directly.

Addition	Cystathionine precursor (μmole total)
None	147
DL-methionine (3·3 mM)	2
DL-threonine (3·3 mM)	147
DL-lysine (3·3 mM)	144

The accumulation of O-succinylhomoserine by Salmonella typhimurium

Smith (1961) has described a series of methionineless mutants of *Salmonella typhimurium*, two of which (strains A15 and B23) appeared unable to form cystathionine. The strains were tested for accumulations by growing them with limiting amounts of homocysteine and chromatographing the concentrated medium on paper chromatograms. The medium from strain B23 contained a compound which ran at R_F 0·35 in *n*-butanol-propionic acid-water and gave a yellow-brown colour with ninhydrin suggesting that it was *O*-succinylhomoserine. Strain A15 did not show any marked accumulation of amino acids. The extent of accumulation by strain B23, when assayed by using extract from *Escherichia coli* strain 2/2, proved to be about 100–150 μmole/l. as compared with up to 2000 μmole/l. for *E. coli* strain 7/9.

To confirm that the compound present in the culture fluid from strain B23 was *O*-succinylhomoserine, organisms were grown on medium supplemented with ^{14}C-succinate, the fluid chromatographed on Dowex-1 resin and samples of the eluate tested for radioactivity. A labelled compound was eluted in the same place as *O*-succinylhomoserine (Fig. 5), and when a sample was run on a paper chromatogram

the isotope ran with synthetic *O*-succinylhomoserine. The accumulation of *O*-succinylhomoserine by strain B23 (like that with strain 7/9) was completely abolished when the organisms were grown with methionine. The absence of the compound was noted when culture fluids were chromatographed on paper; this was confirmed by using ^{14}C-succinate. DL-methionine (3·3 mM) decreased the incorporation of label from succinate to less than 3% (Fig. 5), although growth of the organisms was greater with added methionine.

Synthetic *O*-succinylhomoserine did not support the growth of strains A15 or B23, neither did culture fluid from strain B23 stimulate the growth of strain A15. Thus a Seitz-filtered sample of *O*-succinylhomoserine which contained 0·9 μmoles/ml. (assayed with extract from strain 2/2) had less than 2% of the activity of the same amount of cystathionine for strains A15 and B23.

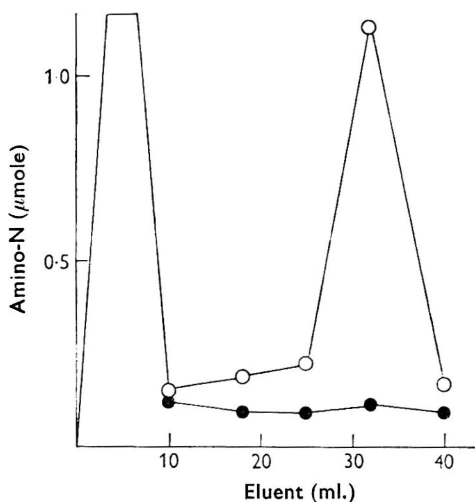


Fig. 4

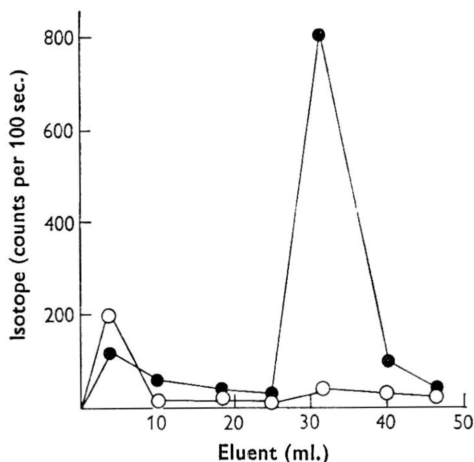


Fig. 5

Fig. 4. The effect of methionine on the accumulation of the cystathionine precursor in the medium of Escherichia coli strain 7/9. Organisms were grown in medium GL with methionine (3·3 mM) present (●) or absent (○). The media were chromatographed on Dowex-1 resin and amino-N assayed with the modified ninhydrin reagent.

Fig. 5. Inhibition by methionine of the accumulation of the cystathionine precursor in the medium of Salmonella typhimurium strain B23. Organisms were harvested from medium GL supplemented with ^{14}C-succinate with methionine (3·3 mM) present (○) or absent (●) and the medium chromatographed on Dowex-1 resin. Radioactivity was measured as described in Methods.

DISCUSSION

Analysis of the compound accumulated by *Escherichia coli* 7/9 showed it contained homoserine and succinate and the reaction with ninhydrin demonstrated the presence of a free amino group. *O*-acyl derivatives of hydroxyamino acids are very unstable to alkali (Sakami & Toennies, 1942) so that *O*-succinylhomoserine was the most probable structure; the behaviour of the compound as a weak acid on Dowex-1 resin was compatible with this structure. Finally, the identity of the compound

with *O*-succinylhomoserine was shown by comparison with the synthetic compound: the two substances behaved alike chromatographically and both gave rise to cystathionine when incubated with cysteine and extract from strain 2/2.

The accumulated *O*-succinylhomoserine was found predominantly in the culture fluid (Table 2) and, since the lysis of dying organisms could only supply a small part of this, the compound must be able to leak out of growing organisms. This is of particular interest since *O*-succinylhomoserine does not support the growth of *Escherichia coli* strain 2/2 although this strain has enzymic mechanisms for the synthesis of cystathionine from it. Although it is possible that the compound is destroyed after entry into the organisms, it seems more likely that this strain is not able to absorb it at all, although the compound is able to leak out of *E. coli* strain 7/9. The accumulation of *O*-succinylhomoserine by cultures of *E. coli* strain 7/9 was not increased by exogenous homoserine or succinate, so that these organisms were able to supply the extra amounts of these compounds then required. The supply of succinate could be readily achieved by increasing the supply of Krebs cycle intermediates, but the extra homoserine synthesis (up to 240 mg./l.) may require increased amounts of the synthesizing enzymes. Although the accumulation of *O*-succinylhomoserine was not increased by supplementing the medium with succinate, as exogenous succinate was very efficiently used for synthesis of *O*-succinylhomoserine, the succinate must be readily equilibrated with the cellular pool used for *O*-succinylhomoserine synthesis. Since succinyl-coenzyme *A* is the active form of succinate used in the reaction (Rowbury & Woods, 1964), the endogenous succinate probably arises from α-oxoglutarate. This is supported by the observation that an auxotroph of *E. coli* which lacks the ability to convert α-oxoglutarate to succinyl-coA requires methionine for growth (Davis *et al.* 1959). Whether the added succinate exerts any effect on succinate synthesis is not certain.

Previously the evidence for the functioning of *O*-succinylhomoserine as a methionine precursor in *Escherichia coli* had been based on its synthesis by extracts of *E. coli* 7/9 from homoserine and succinyl-coenzyme *A* (activity being absent from *E. coli* 2/2) and its conversion to cystathionine by *E. coli* 2/2. (Rowbury, 1962*b*; Rowbury & Woods, 1964). Since strain 7/9 lacks the latter activity, the accumulation of *O*-succinylhomoserine is readily explicable and provides conclusive evidence that the compound is a bona fide methionine precursor. The failure toisolate the precursor from the culture fluid of three other methionine-requiring strains of *E. coli* confirms that the accumulation is a specific result of the metabolic lesion of *E. coli* 7/9. The abolition of accumulation by growth with methionine also implies that the accumulated compound plays an essential role in methionine synthesis and confirms evidence obtained at the enzymic level (Rowbury, 1962*a*) that methionine controls this stage of synthesis. The excessive production of *O*-succinyl homoserine was therefore due to exhaustion of the limited supply of homocysteine.

Smith (1961) showed that certain methionine-requiring mutants of *Salmonella typhimurium* responded to cystathionine, and since wild type *S. typhimurium* has cystathionase activity (Rowbury, 1962*c*) cystathionine seemed to be a precursor of methionine in that organism. This is supported by the isolation of *O*-succinylhomoserine from the culture fluid of the cystathionine-requiring *S. typhimurium* strain B 23. The observation that methionine greatly decreased the accumulation supports the view that the compound is a normal intermediate.

The extent of the accumulations of *O*-succinylhomoserine in *Escherichia coli* and *Salmonella typhimurium* deserves some comment. *S. typhimurium* strain B23 may be more sensitive to the presence of traces of methionine or the enzymes of methionine synthesis may be less active, since the accumulation was much less in this organism. The accumulation of *O*-succinylhomoserine in *E. coli* strain 7/9 must require marked changes in metabolism to provide the amounts of substrates and energy required as the extent of the accumulation greatly exceeds that of methionine precursors in *Neurospora crassa*. Thus one cystathionine-requiring mutant of *N. crassa* (Fischer, 1957) accumulated up to 2·4% cystathionine (of dry wt. organism), while another accumulated 1·5 g. threonine/kg. wet weight (probably about 3% dry wt.) and lesser amounts of homoserine (Fling & Horowitz, 1951) whereas the accumulation of *O*-succinylhomoserine by *E. coli* strain 7/9 was up to 40% of the dry weight.

REFERENCES

Davis, B. D. (1955). Intermediates in amino acid biosynthesis. *Advanc. Enzymol.* **16**, 247.

Davis, B. D., Kornberg, H. L., Nagler, A., Miller, P. & Mingioli, E. (1959). Formation and functions of succinate in *Escherichia coli*. *Fed. Proc.* **18**, 211.

Fischer, G. A. (1957). The cleavage and synthesis of cystathionine in wild type and mutant strains of *Neurospora crassa*. *Biochim. biophys. Acta*, **25**, 50.

Fling, M. & Horowitz, N. H. (1951). Threonine and homoserine in extracts of a methionineless mutant of *Neurospora*. *J. biol. Chem.* **190**, 277.

Gots, J. (1957). Purine metabolism in bacteria. V. Feedback Inhibition. *J. biol. Chem.* **228**, 57.

Guest, J. R., Helleiner, C. W., Cross, M. J. & Woods, D. D. (1960). Cobalamin and the synthesis of methionine by ultrasonic extracts of *Escherichia coli*. *Biochem. J.* **76**, 396.

Harrold, F. M. (1962). Accumulation of cystathionine in a homocysteine requiring mutant of *Aerobacter aerogenes*. *J. Bact.* **84**, 382.

Horowitz, N. H. (1947). Methionine synthesis in *Neurospora*. The isolation of cystathionine. *J. biol. Chem.* **171**, 255.

Moore, S. & Stein, W. H. (1954). A modified reagent for the photometric determination of amino acids and related compounds. *J. biol. Chem.* **211**, 907.

Rowbury, R. J. (1962*a*). Control of cystathionine formation in *Escherichia coli* by methionine. *Biochem. J.* **82**, 24P.

Rowbury, R. J. (1962*b*). A succinyl derivative of homoserine as a precursor of methionine in *Escherichia coli*. *J. gen. Microbiol.* **28**, 5*P*.

Rowbury, R. J. (1962*c*). *Factors influencing the control of methionine synthesis in microorganisms*. D. Phil. Thesis: Oxford University.

Rowbury, R. J. & Woods, D. D. (1961). Further studies on the repression of methionine synthesis in *Escherichia coli*. *J. gen. Microbiol.* **24**, 129.

Rowbury, R. J. & Woods, D. D. (1964). *O*-Succinyl homoserine as an intermediate in the synthesis of cystathionine by *Escherichia coli*. *J. gen. Microbiol.* (in the Press).

Sakami, W. & Toennies, G. (1942). The investigation of amino acid reactions by methods of non-aqueous titrimetry: II. Differential acetylation of hydroxyl groups and a method for the preparation of *O*-acetyl derivatives of hydroxy amino acids. *J. biol. Chem.* **144**, 203.

Smith, D. A. (1961). Some aspects of the genetics of methionineless mutants of *Salmonella typhimurium*. *J. gen. Microbiol.* **24**, 335.

J. gen. Microbiol. (1964), 37, 181–194
Printed in Great Britain

A Study of Features used in the Diagnosis of *Pseudomonas aeruginosa*

By RITA R. COLWELL

Department of Biology, Georgetown University, Washington, D.C. 20007, U.S.A.

SUMMARY

Thirty-three strains of *Pseudomonas aeruginosa*, previously described as a species group by a quantitative study, were subjected to an extended schedule of tests used in bacterial classification and identification. Adansonian analysis by electronic computer confirmed previous results, indicating that the 33 strains should be treated as a species group. The 172 features coded for each strain were analysed by computer, yielding a 'probability of occurrence' of each feature within the species, *P. aeruginosa*. This output was then used to select features appropriate for diagnostic purposes. From the analyses it was also possible to measure the sensitivity of several methods for determining the presence or absence of characteristics such as pigment production and oxidation of gluconate to ketogluconate. The general applicability of the use of the computer to cooperative pooling of data by bacteriologists is discussed.

INTRODUCTION

The incidence of *Pseudomonas aeruginosa* in clinical infections and in various natural environments, coupled with the difficulty of diagnosis of this micro-organism, has led to an increased interest in its identification and classification (Ringen & Drake, 1952; Gould & McLeod, 1960; Rogers, 1960; Pulverer & Korth, 1962; Curtin, Petersdorf & Bennett, 1961). A large number of papers have been published concerning *P. aeruginosa*, particularly on methods for the selective isolation of the organism, methods for enhancement of pigment production and new tests for classification (Seleen & Stark, 1943; Bühlmann, Vischer & Bruhin, 1961*a*, *b*). Several tests have been proposed as specific for *P. aeruginosa* (Haynes, 1951; Gaby & Free, 1953, 1958; Kovacs, 1956). A comparative study of the tests suggested for identification of *P. aeruginosa* should be useful for those workers who isolate this micro-organism frequently in routine studies. Heretofore, the difficulty in carrying out a study of this type lay in the handling and correlation of the large amount of necessary data. For such problems, electronic computers are a valuable bacteriological tool. Diagnostic characters can be extracted from the total taxonomic data for given groups of micro-organisms and the relative merits of the characteristics considered.

Several years ago, the application of electronic computers to bacterial taxonomy was studied by an analysis of data for a number of pseudomonads, both culture collection strains and fresh isolates (Colwell & Liston, 1961*a*, *b*; Rhodes, 1961). 'Taxonomic clusters' within the genus *Pseudomonas* were identified. Subsequently, using 33 strains from the American Type Culture Collection, a species cluster was defined for *P. aeruginosa*, and a quantitative basis for species definition was

established (Liston, Wiebe & Colwell, 1962, 1963). As the next logical step, it appeared appropriate to increase the battery of tests as widely as possible, thereby extending the description of *P. aeruginosa* and providing a means of determining the reliability of the various tests proposed for identification and classification of the organism. Furthermore, the data so obtained could be mathematically sifted by the computer to extract significant diagnostic features. The present paper describes a study of the characteristics used in diagnosis of *P. aeruginosa* and of the application of the electronic computer as an aid in classifying this species.

METHODS

The organisms studied were 33 strains of *Pseudomonas aeruginosa* received from the American Type Culture Collection and treated previously in a quantitative study by electronic computer as a species group (Liston *et al.* 1963); see Table 1.

Methods used were those described by Colwell & Liston (1961*a*) or the Society of American Bacteriologists (1957), except as indicated below. The standard inoculum for all tests was a single drop (1/20 ml.) from a sterile disposable pipette (Fisher Scientific Company) of a 24–48 hr broth culture. The stock culture medium (YE) was a modification of that used by Rhodes (1959): Difco yeast extract, 0·3 g.; Difco Bacto-Proteose Peptone, 1·0 g.; NaCl 0·5 g.; agar 1·5 g. (agar omitted from liquid stock media); distilled water 1 l.; adjusted to pH 7·2–7·4 with NaOH. Tests were carried out at room temperature (25°) except where otherwise indicated.

Effect of environmental conditions on growth

Temperature range of growth. The ability of strains to grow on YE agar slopes and in YE broth at 0°, 5°, 10°, 15°, 25°, 30°, 35° and 40° was screened by incubation of the inoculated media in incubator rooms temperature-controlled to ± 0·5°, and the upper and lower limits of growth determined by incubation in a water bath at the desired temperature. As suggested by Haynes & Rhodes (1962), a strain was considered incapable of growth at a given temperature unless it could survive at least three serial transfers at that temperature. In addition, where possible, the medium was pre-incubated at the test temperature for 6–12 hr before inoculation. The incubation period extended from 5 days to 4 weeks, depending on the temperature of incubation.

Salinity range of growth. The strains were inoculated into YE broth tubes, with final NaCl concentrations of 0, 0·5, 3·0, 5·0 and 10·0 %. The tubes were incubated for 14 days after inoculation.

pH range of growth. YE broth, adjusted to pH 4·0, 4·5, 5·0, 5·5, 6·0, 7·0, 8·0 and 9·0, with HCl or NaOH as required, was inoculated and incubated for 14 days, growth being observed at 1, 2, 7 and 14 days.

Sensitivity to antibiotics and a 'vibriostatic compound'. The strains were tested for sensitivity to penicillin (high concentration), dihydrostreptomycin (10 μg.), chloramphenicol (30 μg.), chlortetracycline (30 μg.), oxytetrocycline (30 μg.), polymyxin B (300 units), tetracycline (30 μg.; Bauer, Roberts & Kirby, 1959–60) and the vibriostatic compound (2,4-diamino-6,7-diisopropylpteridine) described by Shewan, Hodgkiss & Liston (1954). YE agar plates were used, with sensitivity discs (Baltimore Biological Laboratory, Inc.) for the antibiotics listed.

Physiological tests

Glucose utilization. The method of Hugh & Leifson (1953) was used to determine whether glucose was used oxidatively or fermentatively. The miniature tube method (Colwell & Quadling, 1962) was also used. Growth in glucose with and without added iodoacetate (Lysenko, 1961) was tested.

Production of acid from carbohydrate. Cultures were incubated for 4 weeks in a bromcresol-purple broth basal medium (Difco Laboratories) with 1·0 % filter-sterilized test carbohydrate added. Glucose, lactose, maltose, sucrose, galactose, mannitol, mannose, melibiose, melezitose, dextrin, glycerol and sorbitol were used. In addition, the Hugh & Leifson (1953) basal medium without agar and with Durham cubes was used, with added filter-sterilized glucose, lactose or maltose as test carbohydrates. Each culture was examined at 1, 7, 14 and 28 days for acid and gas production in all tests.

Hydrolysis of aesculin. The agar plate technique of Sneath (1956) was used.

Production of 2-ketogluconic acid. Cultures were incubated for 7 days in Paton's broth medium (Paton, 1959); the presence of reducing sugars was determined by adding Benedict's reagent (Clinitest tablet, Ames Co. of Canada, Ltd., Toronto). The method of Haynes (1951) was also used and the production of slime and 'oyster' formation noted. The rapid test for gluconate oxidation (Gaby & Free, 1958) was done with gluconate substrate tablets (Key Scientific Products Inc., Los Angeles, U.S.A.) but the incubation time was increased to 72 hr at room temperature on a rotary shaker.

Oxidation of calcium lactate through acetate to carbonate and production and accumulation of dihydroxyacetone in media containing glycerol and the production of acetic acid from ethanol were tested by the methods given by Shimwell, Carr & Rhodes (1960).

Production of pigment. Several media, designed for, or described as enhancing, pigment production by *Pseudomonas aeruginosa* were tested. All the media used were scored for production of a green water-soluble pigment. Media designed for production of pyocyanine (Burton, Eagles & Campbell, 1947; Burton, Campbell & Eagles, 1948; King, Ward & Raney, 1954), pyorubin (Meader, Robinson & Leonard, 1925; King, Ward & Raney, 1954), and fluorescin (King, Campbell & Eagles, 1948; King, Ward & Raney 1954; Paton, 1959), were prepared as described by these authors, inoculated and incubated up to 7 days, being routinely examined for the production of visible pigment or pigment fluorescence under ultraviolet radiation in the case of fluorescin. Pigment production was tested for on Sabouraud maltose agar (Martineau & Forget, 1958). Pyocyanine and pyorubin were identified by the presumptive tests outlined by Wetmore & Gochenour (1956). Finally, sporadic production of oxychlororaphin and chlororaphin crystals was noted but, as observed by Haynes & Rhodes (1962), no single medium served as indicator for consistent production of these compounds.

Ammonia production was tested for by Nessler's reagent after incubation for 7 days in YE broth.

Hydrogen sulphide formation from sodium thiosulphate and from cystine was tested by the miniature tube method of Colwell & Quadling (1962). Also used, for comparative purposes, were the Difco lead acetate agar slopes.

Trimethylamine oxide reduction. Cultures were incubated for 14 days in Wood & Baird's medium (1943) and the presence of trimethylamine was then tested for according to the method given by the authors.

Hydrolysis of Tween 20, 40, 60 *and* 80. The use of the water-soluble Tweens for easy detection of lipolytic activity was described by Sierra (1957). His technique, using 1·0 % Tween 20, 40, 60 or 80 in calcium chloride peptone agar, was followed. Cultures were kept for 14 days, being examined routinely for the appearance of conspicuous precipitates around the colonies.

Catalase, cytochrome oxidase and oxidase formation. Catalase was tested for by adding a drop of ('10 vol.') hydrogen peroxide to a smear of a 24–48 hr YE agar colony on a glass slide; for cytochrome oxidase and oxidase formation the methods of Gaby & Free (1958) and Kovacs (1956), respectively, were used.

De-amidation of acetamide was tested for by the method outlined by Bühlmann, Vischer & Bruhin (1961*b*).

Lecithinase was tested for by spotting a drop of a 24–48 hr YE broth culture on to an egg-yolk agar plate consisting of Difco Nutrient Agar containing 10 % (v/v) egg-yolk suspension. The egg-yolk suspension was prepared as follows: an egg yolk was washed in 0·2 % NaCl, placed in a Blendor jar with 200 ml. 0·2 % NaCl and the resulting suspension centrifuged at 13,000 rev./min. for 5 min. in a high-speed refrigerated centrifuge. The supernatant fluid was sterilized by Seitz filtration and added to cooled sterile Difco Nutrient Agar before pouring plates.

Arginine dihydrolase was tested for by the method of Thornley (1960).

Melanin production from tyrosine and from phenylalanine was observed by the method of Brisou & Menantaud (1957).

Production of phenylpyruvic acid from phenylalanine was tested for by the method of Ewing, Davis & Reavis (1957).

Nutrition

The standard inoculation for all the tests was carried out by spotting agar plates containing basal salts medium (NaCl 5·0 g.; $MgSO_4.7H_2O$, 0·2 g.; K_2HPO_4, 1·0 g.; distilled water 1 l.; adjusted pH 6·8) with added organic acids and ammonium-N or amino acids. A control plate with only the basal salts medium was tested at the same time. Vitamin-free Casamino acids, proline, DL-alanine, β-alanine, arginine, lysine, phenylalanine (California Biochemical Corp.) and sodium formate were tested for ability to support growth.

Computer analysis

The results of each test were recorded and coded, yielding 172 coded characteristics per strain. These data were transferred to IBM punch cards and were processed by electronic computer. Similarity values were obtained and sorted as described previously (Colwell & Liston, 1961*a*). The data for the 33 strains were then processed by electronic computer so that the frequency of occurrence of each feature in the group was computed by using the programmed formula $v = n/N$, where n = the number of strains showing the characteristics and N = the total number of strains in the cluster. The output, sorted by decreasing frequency, was then compiled, grouping characteristics into morphological, cultural, etc., feature clusters.

The IBM 1620 40,000 K electronic computer was used in these studies and the programs have been documented (Colwell, in Taxometrics no. 3).

RESULTS AND DISCUSSION

Quantitative analysis of the species group

Previous study indicated that all strains listed in Table 1, with the exception of 257, 7701 and 9027, can be considered members of the species *Pseudomonas aeruginosa* (Liston *et al.* 1963). Seventy-eight coded features, representing 134 character states, were used to describe and quantify the species. The $\overline{\Delta S}$ for the 30 strains comprising the group was 77%. Other parameters were: $\bar{N}_s = 31$; $\bar{N}_d = 9$; $\bar{N}_0 = 38$ and $\bar{M} = 88\%$, where N_s = number of shared features, N_d = number of features present in one strain and not the other, and M = matching coefficient, or similarity index in which the number of concordances between negative responses as well as positive responses is taken into account (Silvestri, Turri, Hill & Gilardi, 1962). In the study described in this paper, the number of coded features was increased to 172 for each strain tested and the calculations repeated, giving: $\overline{\Delta S} = 82\%$; $\bar{N}_s = 84$;

Table 1. *American Type Culture Collection numbers of Pseudomonas aeruginosa strains used in this study**

97	10145	14209
257	10197	14210
260	10752	14211
262	13388	14212
7700	14149	14213
7701	14203	14214
8689	14204	14215
8707	14205	14216
8709	14206	14217
9027	14207	14218
8721	14208	14219

* Additional information for each strain is given by Liston, Wiebe & Colwell (1963).

Table 2. *Similarity of each strain to the calculated median organism*

Organism (ATCC no.)	S value with median	Organism (ATCC no.)	S value with median
14214	93		
14216	92	7701	89
14203	92	14213	88
14149	92	8707	88
8689	91	97	87
14208	91	14219	87
14209	91	14210	87
14215	91	10145	86
7700	91	10752	86
14218	91	9027	86
14204	90	14212	86
14217	90	9721	85
13388	90	14207	85
260	89	257	84
14205	89	8709	84
14206	89	10197	82
14211	89	262	70

$\bar{N}_d = 19$; $\bar{N}_0 = 69$ and $\overline{M} = 89\%$. The values obtained were comparable to those from the previous published calculations except that now higher similarity indices resulted for strain/strain comparisons and the intra-group calculations.

Listed in Table 2 are the similarity indices for each strain compared with the hypothetical Median Organism which was derived according to the method of Liston *et al.* (1963). Using their '75 % rule', strain 262 would not be included in the species group. The question of whether to include the strains 9027, 7701, 257 and 262 in the *Pseudomonas aeruginosa* species was considered in the earlier published study and the conclusion drawn was that 9027, 7701 and 257 should be excluded. From the data listed in Table 2, only strain 262 would now be considered of doubtful membership. However, the exclusion of strain 262 does not effect a very large change in the values listed above. Recalculated, these were: $\overline{\Delta S} = 83\%$; $\bar{N} = 85$; $\bar{N}_d = 18$; $\bar{N}_0 = 69$ and $\overline{M} = 90\%$.

Analysis of features

Using the *Pseudomonas aeruginosa* strains as a quantitatively defined species group, a description of the species can be derived which may be assumed to approximate to the population phenotype. A listing of the features in terms of frequency of occurrence should provide a best estimate of the probability of occurrence of the various characters within the species. In other similar samplings these data may then be useful in the identification and classification process. The compiled output of features and frequency of occurrence, condensed according to operational fields, viz. morphology, physiology, etc., is given in Tables 3–7.

Cultural characteristics of the strains are given in Table 3. All strains were Gram-negative rods with polar flagella, occurring as singles or pairs. Slender, round or tapered organisms of short to medium length were observed, with no curved, oval-shaped organisms or chains scored. The characteristics of growth in YE broth were variable, with moderate even turbidity most frequent, granular turbidity rare, and pellicle formation occurring with a frequency of 0·48.

The majority of the cultures formed grey, flat, entire, translucent colonies, about 2–5 mm. in diameter on YE agar, but there were opaque, off-white colonies occurring at low frequency. Variation in the colonial morphology of *Pseudomonas aeruginosa* has long been known (Hadley, 1937) and has been studied by several investigators (Gaby, 1946; Gaby & Free, 1953; Williamson, 1956). The observations made in the present work confirm the results of other investigators that colonies on agar may be exclusively of one type or a mixture of both types. Attempts to correlate pathogenicity with the colonial variants have been of relatively limited value. However, serological studies of the species *P. aeruginosa* have depended largely on selection of stable smooth colonies for propagation of antigens (Verder & Evans, 1961).

The effect of temperature, pH value and salt concentration on growth are among the coded features included in Table 3. In this study, all cultures grew at temperatures ranging from 25° to 41°; none was able to grow at temperatures from 0° to 5°. Thus, the full temperature range of growth was 5·5–45°. However, it should be stated that the majority of strains grew at temperatures ranging from 8° to 42°. On the basis of the data given, the test for growth at 42° suggested by Haynes (1951)

for the diagnosis of *Pseudomonas aeruginosa* is not absolute but is a feature which occurs with sufficiently high frequency to justify its use in a diagnostic procedure.

Growth was observed in YE broth media in the range pH 4·0–9·0, most strains growing in the YE broth initially in the range pH 5·0–9·0. Sodium chloride concentrations, ranging from none added to 5 % sodium chloride were tolerated by all but one strain.

Table 3. *Feature and frequency of occurrence within the Pseudomonas aeruginosa species cluster: cultural characteristics*

YE broth					
Rods	1	Motile by polar flagella	0·94	Ovals	0
Singles	1	Tapered	0·88	Stout (0·6–1·0 μ)	0
Pairs	1	Short (0·2–0·6 μ)	0·76	Chains	0
Gram-negative	1	Round end	0·58	Gram-variable	0
		Slender (0·2–0·6 μ)	0·42		
		Medium length (0·6–1·2 μ)	0·30		
		Curved rods	0·06		
		'Filaments'	0·03		
		Even turbidity	0·85	Heavy turbidity	0
		Moderate turbidity	0·70		
		Pellicle	0·48		
		Slight turbidity	0·30		
		Granular turbidity	0·12		
		Ring	0·12		
YE agar					
		Grey	0·85	White	0
		Entire	0·82	Convex	0
		Medium (2–5 mm) colony	0·85		
		Translucent	0·73		
		Opaque	0·30		
		Small (< 2 mm) colony	0·18		
		Off-white	0·15		
Growth at					
25°–41°	1	42°	0·97	0°–5°	0
		44°	0·73		
		15°	0·97		
		10°	0·97		
		8°	0·91		
		7°	0·79		
		6°	0·07		
Growth at					
pH 5·5–9·0	1	pH 5·0	0·97		
		pH 4·5	0·30		
		pH 4·0	0·06		
Growth in					
0·5 % NaCl	1	0 % NaCl	0·97	10 % NaCl	0
		3·0 %–5·0 % NaCl	0·97		

Several media were devised in an attempt to determine a nutritional pattern for the species. Single amino acids in a buffered salts agar basal medium were tested for ability to support growth. Results of studies with amino acids are given in Table 4. All strains grew in vitamin-free Casamino acids, buffered salts agar basal medium and in the basal medium to which the amino acids, proline, β-alanine, DL-alanine, phenylalanine, lysine and arginine, separately, had been added. Less than half the strains were able to utilize lysine as sole nutrient source, although all

strains produced abundant growth in the lysine + peptone broth medium of Falkow (1958). Growth on phenylalanine agar was rare (0·03) and sparse when it did occur. The test for phenylalanine deaminase (Ewing *et al.* 1957) was negative for all strains.

Table 4. *Feature and frequency of occurrence within the Pseudomonas aeruginosa species cluster: nutritional characteristics and antibiotic sensitivities*

Growth in					
Koser's citrate medium	1	0·3 % malonate	0·97	0·1 % sodium formate	0
Simmon's citrate medium	1	0·1 % DL-alanine + salts	0·97		
Vitamin-free Casamino acids	1	0·1 % β-alanine + salts	0·97		
		0·1 % arginine + salts	0·97		
Lysine broth (Falkow, 1958)	1	1·0 % calcium lactate (oxidation of calcium lactate through acetate to carbonate)	0·94		
0·1 % proline + salts*	1				
$NH_4H_2PO_4$ + salts	1				
		0·1 % lysine + salts	0·45		
		0·1 % phenylalanine + salts	0·03		
Sensitive to					
		Oxytetracycline 30 µg.†	0·97	Erythromycin 15 µg.	0
		Polymyxin B 300 units	0·91	Penicillin high concn.	0
		Tetracycline 30 µg.	0·85	Pteridin compound	0
		Kanamycin 30 µg.	0·78		
		Dihydrostreptomycin 10 µg.	0·73		
		Chlortetracycline 30 µg.	0·45		
		Chloramphenicol 30 µg.	0·15		
		Novobiocin 30 µg.	0·06		

* g/l.: NaCl, 5·0; $MgSO_4.7H_2O$, 0·2; K_2HPO_4, 1·0; pH 6·8.
† Sensitivity discs, Baltimore Biological Laboratory, Inc.

Other compounds were tested for ability to support growth and several antibiotics were tested for growth inhibition (Table 4). All strains were citrate-positive in both Koser's and Simmons's media. This, of course, suggested that ammonium-N was a suitable source of nitrogen for growth, and was verified by the observation that all strains were able to grow on a basal medium to which $NH_4H_2PO_4$ had been added.

Growth in a 0·3 % malonate broth (Ewing, 1962) and oxidation of calcium lactate to carbonate (Shimwell *et al.* 1960) were features which occurred at very high frequencies within the group.

Of the antibiotics which were used (Table 4), all strains tested were resistant to penicillin, erythromycin and the pteridine compound. A majority of the strains tested were resistant to novobiocin and chloramphenicol. The antibiotics, oxytetracycline, polymyxin B, tetracycline, kanamycin, and dihydrostreptomycin, were inhibitory for > 70 % of the strains. Sensitivity to chlortetracycline occurred at a frequency of 0·45. From figures given in Table 4, an estimate of antibiotic sensitivities for strains of *Pseudomonas aeruginosa* can be determined.

Reactions in various carbohydrate media were examined (Table 5). Attack on carbohydrates was oxidative when both the methods of Hugh & Leifson (1953) and Colwell & Quadling (1962) were followed. There was no fermentation by any of the 33 strains. Acid without gas was produced from glucose, and iodoacetate did not inhibit this acid production. Of the carbohydrates tested, only glucose (0·97) and

galactose (0·40) were utilized by many of the strains. Acid production from mannose, melezitose, glycerol and sucrose were features of infrequent occurrence. Positive methyl-red and Voges-Proskauer reactions and hydrolysis of aesculin were also features of rare occurrence in the group. Dihydroxyacetone was not accumulated from glycerol by any of the strains, nor was acetic acid produced from ethanol. Starch was not hydrolysed.

Table 5. *Feature and frequency of occurrence within the Pseudomonas aeruginosa species cluster: carbohydrate reactions*

Feature	Frequency	Feature	Frequency	Feature	Frequency
Growth in glucose+iodo-acetate medium	1	Glucose oxidized in presence iodoacetate	0·97	Glucose fermented (Hugh & Leifson)	0
		Glucose oxidized (Hugh & Leifson	0·97	Glucose fermented (miniature tube method)	0
		Glucose oxidized (miniature tube method)	0·97	Gas from carbo-hydrates	0
		Gluconate → ketogluconate (Haynes)	0·97	Acid from lactose	0
		Gluconate → ketogluconate (Gaby & Free)	0·92	Acid from maltose	0
		Gluconate → ketogluconate (Paton)	0·94	Acid from mannitol	0
		Slime production in glu-conate medium (Haynes)	0·88	Acid from melibiose	0
		Acid from galactose	0·40	Acid from sorbitol	0
		Acid from mannose	0·17	Acid from dextrin	0
		Acid from melezitose	0·03	Starch hydrolysed	0
		Acid from glycerol	0·03	Glycerol → dihydroxy-acetone	0
		Acid from sucrose	0·03	Ethanol → acetic acid	0
		Methyl-red positive	0·03	Agar digested	0
		Voges-Proskauer positive	0·03		
		Aesculin hydrolysed	0·03		

Three methods for detection of gluconate oxidation were used and the results are given in Table 5. Most of the strains were able to produce ketogluconate from gluconate substrate. The technique of Gaby & Free (1958) was quick and simple to carry out, but the chance of not detecting a positive reaction was greater than with the method proposed by Haynes (1951).

Additional data obtained from other biochemical tests are given in Table 6. All strains produced ammonia in peptone water, reduced nitrates and nitrites, and showed catalase, cytochrome oxidase, oxidase, acetamide deamidase, lipase and lecithinase. The presence of urease and arginine dihydrolase were features which occurred at significantly high frequencies. Acid peptonization of litmus milk, hydrolysis of casein and production of hydrogen sulphide from cystine were characters of frequent occurrence. Gelatin liquefaction was somewhat less constant a feature (0·61). Characteristics such as melanin production from tyrosine and indole production from tryptophan were features of low occurrence, indicating a sporadic incidence in the group. A trimethylamine oxide reductase was not detected.

Certain features such as production of hydrogen sulphide depend greatly on the medium and method used. The lead acetate agar technique cannot be considered satisfactory for detection of sulphide production from proteins or their split products, at least for pseudomonads. Other investigators have commented on the inadequacies of the lead acetate agar method (Sherwood, Johnson & Radotincky, 1926; Rhodes,

1959). As seen from Table 6, the miniature tube method of Colwell & Quadling (1962) is more suitable for the detection of hydrogen sulphide. Hydrogen sulphide production from sodium thiosulphate, as examined by the miniature tube method, was not a characteristic of the 33 strains of *Pseudomonas aeruginosa* examined.

Table 6. *Feature and frequency of occurrence within the Pseudomonas aeruginosa species cluster: additional biochemical characteristics*

Feature	Frequency
Ammonia production in peptone water	1
Nitrate reduced to nitrite	1
Nitrite reduced	1
Catalase present	1
Oxidase present (Kovacs, 1956)	1
Acetamide deamidated	1
Lipase present (Tween 40, 60, 80)	1
Lecithinase present	1
Cytochrome oxidase present (Gaby & Free, 1953)	0·97
Urease present	0·97
Arginine dihydrolase present	0·97
Lipase (Tween 20)	0·97
Litmus milk peptonized	0·94
Litmus milk acid	0·91
Casein hydrolysed	0·85
H_2S produced from cystine	0·82
Gelatin liquefied	0·61
H_2S produced on lead acetate agar	0·21
Litmus milk surface peptonized only	0·21
Tyrosine → melanins	0·18
Tryptophan → indole	0·17
Litmus milk alkaline	0·06
H_2S produced from sodium thiosulphate	0·06
Trimethylamine oxide reduced to trimethylamine	0
Litmus milk reduced	0
Litmus milk acid → alkaline	0
Litmus milk alkaline → acid	0
Phenylalanine → phenylpyruvic acid	0
Phenylalanine → melanins	0

Pigmentation, a 'key' feature in some classificatory schema, was examined rather carefully. Five different media, cited as specific for pigment production by *Pseudomonas*, were compared. The results are given in Table 7. In YE broth and on YE agar, both containing Difco Proteose Peptone, and on King, Campbell & Eagles (1954) agar medium, a green diffusible pigment was observed for all strains tested. The Sabouraud maltose agar, suggested by Martineau & Forget (1958) and Davis, Sellers, Orbach & Weddington (1960), was not as efficient for determining pigment production as either YE medium or the King *et al.* medium. However, not all pigments which were produced by these strains were pyocyanine; a green pigment which was chloroform-soluble and pH-sensitive, for the purposes of this study, was considered to be pyocyanine. Such a pigment was detected in 97% of the strains tested, but the frequency with which it was observed varied with the medium used (0·79–0·97). Similarly, the pigment fluorescin occurred with equally variable frequency among the strains (Table 7). The King *et al.* media designated for enhanced production of the pigments pyocyanine, pyorubin and fluorescin, gave less equivocal and somewhat more reliable results than the other media used.

Pigment production by *Pseudomonas aeruginosa* is considered an important diagnostic feature when it is present in a culture, but non-pigmented strains occur and create a difficult problem for the diagnostician who relies on 'key' features (Gaby & Free, 1958; Verder & Evans, 1961; Bühlman *et al.* 1961*b*). The variability of pigment production among strains of *P. aeruginosa* has long been known (Baerthlein, 1918; Charrin & Phisalix, 1892; Eisenberg, 1914). Requirements for pyocyanine, pyorubin and fluorescin production by *P. aeruginosa* have been studied

by a number of workers since Gessard's early publications on the 'pyocyanogenic bacillus' (1890, 1891, 1892, 1918). The type of peptone used (Georgia & Poe, 1932) and the inorganic salts present in the medium (Burton *et al.* 1948) have been shown to influence the amount and kind of pigment which is produced. This single feature, or any other feature whether 'computed' or designated arbitrarily, should not be relied upon as sole criterion for diagnosis.

Table 7. *Feature and frequency of occurrence within the Pseudomonas aeruginosa species cluster: pigmentation characteristics*

Green, diffusible, water-soluble pigment produced in			
YE agar	1	Sabouraud maltose agar	0·88
King *et al.* agar	1	Burton *et al.* medium	0·82
Production of specific pigments			
		Pyocyanine—King *et al.* agar	0·97
		Pyocyanine—Burton *et al.* medium	0·79
		Pyorubin—King *et al.* agar	0·79
		Fluorescin—King *et al.* agar	0·94
		Fluorescin—YE broth	0·90
		Fluorescin—Paton's medium	0·88
		Fluorescin—Burton *et al.* medium	0·70
		Oxychlororaphine	0·12

Since no single character can be selected as 'non-mutable', hence an essential prerequisite for membership in a given taxon (a monothetically as opposed to a polythetically constructed taxon), how can a diagnosis be made with high degree of confidence and minimum investment of labour and material? Obviously, the problem is one of equilibration of input and output information until an optimum for both is reached. Certainly it is safe to say that maximum information, that is, all possible information, about a given strain would result in an identification and classification of maximum confidence estimate, which is absolute certainty. Since it is not always possible or desirable to determine even 172 coded features for every new isolate, the question then is, how many features and what combination of these are needed for identification and classification? Proceeding from the data given, the description of *Pseudomonas aeruginosa* would be as follows: a Gram-negative, rod-shaped, polarly-flagellated organism, occurring as single organisms and in pairs, with no oval-shaped organisms or chains; which formed grey, flat, entire translucent colonies about 2–5 mm in diameter on YE agar medium; which grew at temperatures of 25–41° (but not at 0–5°) and at pH 5·0–9·0; tolerated NaCl concentrations up to 5 %; was able to grow in an inorganic-N sodium citrate medium, or on a basal salts medium to which 0·1 % proline or vitamin-free Casamino acids was added, and in a glucose medium with added iodoacetate; did not ferment glucose or produce acid from lactose, maltose, mannitol, melibiose, sorbitol, dextrin; did not hydrolyse starch, digest agar, produce ethanol from acetic acid or accumulate dihydroxyacetone from glycerol; which produced ammonia in peptone water; reduced nitrate and nitrite; produced catalase, oxidase, acetamide deamidase, lipase and lecithinase but not trimethylamine oxide reductase and phenylalanine deaminase; finally, which produced a green water-soluble pigment on YE agar. Yet no single character of those listed is either indispensable in defining the organism or is a sufficient single criterion.

In fact, then, the simultaneous possession of a group of characters of high frequency of occurrence among strains of a given species is that which is definite (Liston *et al.* 1963). Moller (1962) proceeded by a similar line of reasoning, and introduced the concept of the attribution decision, a decision determined by the probability of belonging of each element to one or more taxa with respect to each classification criterion. However, it may be more useful to deal with the taxonomic data available for bacterial species in terms of frequency of occurrence within the cluster of organisms as the estimate of reliability of classification, the 'probability of belonging' to a taxon increasing with increasing occurrence of characters of high frequency for that taxon.

Features found to have a very high probability of occurrence taken together with features of very low probability of occurrence provide the convenient diagnostic keys for recognition of additional isolates belonging to the species. It is possible, of course, to minimize the number of necessary attributes since, the more attributes which appear unique to the species, fewer are then required to define it. Conversely, those features of low frequency in the taxon will decrease the 'probability of belonging' for a test organism. Although these attributes may be rare for the taxon they should not be used to exclude strains from the group, except, of course, when many rare characters occur together. Furthermore, from the computed relationships to the hypothetical median organism, such as for the strains studied, it is possible to select a working neotype (Colwell, 1964).

The storing, sorting and sifting of taxonomic data by machine can be extremely useful for diagnosis as well as construction of taxa, that is, for selection of diagnostic features, comparison of methods, identification and choosing neotype strains. The total amount of data currently available for any single microbial group would be handled easily by computers presently installed in university, government and industrial laboratories. A co-operative study and subsequent data analysis may offer objective evaluation of diagnostic procedures currently followed in most laboratories.

This investigation was supported by a grant (G-19405) from the U.S.A. National Science Foundation. Appreciation is expressed to Dr N. E. Gibbons for assistance in obtaining laboratory and computer facilities during tenure as Guest Scientist at the National Research Council of Canada where part of the research was carried out, and to Dr W. A. Clark of the American Type Culture Collection for gift of the *Pseudomonas aeruginosa* strains.

REFERENCES

BAERTHLEIN, K. (1918). Über bakterielle Variabilität, insbesondere sogennannte Bakterienmutationen. *Zbl. Bakt.* (1 *Abt. Orig.*), 81, 428.

BAUER, A. W., ROBERTS, C. E. JR. & KIRBY, W. M. M. (1959–60). Single disc versus multiple disc and plate dilution techniques for antibiotic sensitivity testing. *Antibiotics*, 1959–1960, p. 574.

BRISOU, J. & MENANTAUD (1957). Contribution à l'étude des tyrosinases microbiénnes. *Bull. Ass. Dipl. Microbiol., Nancy*, 67, 3.

BÜHLMANN, X., VISCHER, W. A. & BRUHIN, H. (1961*a*). Die Identifizierung nicht Pyocyanin-bildender Stämme von *Pseudomonas aeruginosa*. *Zbl. Bakt.* (1. *Abt. Orig.*), **183**, 368.

Bühlmann, X., Vischer, W. A. & Bruhin, H. (1961*b*). Identification of apyocyanogenic strains of *Pseudomonas aeruginosa*. *J. Bact.* **82**, 787.

Burton, M. O., Campbell, J. J. R. & Eagles, B. A. (1948). The mineral requirements for pyocyanin production. *Can. J. Res.* **26**, 15.

Burton, M. O., Eagles, B. A. & Campbell, J. J. R. (1947). The amino acid requirements for pyocyanin production. *Can. J. Res.* **25**, 121.

Charrin & Phisalix (1892). Abolition persistante de la fonction chromogène du *Bacillus pyocyaneus*. *C.r. Séanc. Soc. Biol., Paris*, **114**, 1565.

Colwell, R. R. (1964). Proposal of a neotype, ATCC 14216, for *Pseudomonas aeruginosa* (Schroeter, 1872) Migula 1900 and request for an opinion. *Int. Bull. bact. Nomencl.* (in the Press).

Colwell, R. R. & Liston, J. (1961*a*). Taxonomic relationships among the pseudomonads. *J. Bact.* **82**, 1.

Colwell, R. R. & Liston, J. (1961*b*). Taxonomic analysis with the electronic computer of some *Xanthomonas* and *Pseudomonas* species. *J. Bact.* **82**, 913.

Colwell, R. R. & Quadling, C. (1962). Miniature tubes in diagnostic bacteriology. *Can. J. Microbiol.* **8**, 813.

Curtin, J. A., Petersdorf, R. G. & Bennett, I. L. (1961). Pseudomonas bacteremia: review of ninety-one cases. *Ann. intern. Med.* **54**, 1077.

Davis, I., Sellers, W., Orbach, H. & Weddington, G. (1960). An evaluation of several media for the early detection of *Pseudomonas aeruginosa* encountered in clinical practice. *J. lab. clin. Med.* **55**, 139.

Eisenberg, P. (1914). Untersuchungen über die Variabilität der Bakterien. *Zbl. Bakt.* (1 *Abt. Orig.*), **73**, 466.

Ewing, W. H. (1962). Enterobacteriaceae. Biochemical methods for group differentiation. *Publ. Hlth Serv. Publ., Wash.* no. 734.

Ewing, W. H., Davis, B. R. & Reaves, R. W. (1957). Phenylalanine and malonate media and their use in enteric bacteriology. *Publ. Hlth Lab.* **15**, 153.

Falkow, S. (1958). Activity of lysine decarboxylase as an aid in the identification of salmonellae and shigellae. *Amer. J. clin. Path.* **29**, 598.

Gaby, W. L. (1946). A study of the dissociative behaviour of *Pseudomonas aeruginosa*. *J. Bact.* **51**, 217.

Gaby, W. L. & Free, E. (1953). The occurrence and identification of non-pigmented strains of *Pseudomonas aeruginosa* in the clinical laboratory. *J. Bact.* **65**, 746.

Gaby, W. L. & Free, E. (1958). Differential diagnosis of *Pseudomonas*-like microorganisms in the clinical laboratory. *J. Bact.* **76**, 442.

Georgia, F. R. & Poe, C. F. (1932). Study of bacterial fluorescence in various media. II. The production of fluorescence in media made from peptones. *J. Bact.* **23**, 135.

Gessard, C. (1890). Nouvelles recherches sur le microbe pyocyanique. *Ann. Inst. Pasteur*, **4**, 88.

Gessard, C. (1891). Des races du bacille pyocyanique. *Ann. Inst. Pasteur*, **5**, 65.

Gessard, C. (1892). Sur la fonction fluorescigène des microbes. *Ann. Inst. Pasteur*, **6**, 801.

Gessard, C. (1918). Technique d'identification des germes pyocyaniques. *Ann. Inst. Pasteur*, **34**, 88.

Gould, J. C. & McLeod, J. W. (1960). A study of the use of agglutinating sera and phage lysis in the classification of strains of *Pseudomonas aeruginosa*. *J. Path. Bact.* **79**, 295.

Hadley, P. (1937). Further advances in the study of microbic dissociation. *J. infect. Dis.* **60**, 129.

Haynes, W. C. (1951). *Pseudomonas aeruginosa*—its characterization and identification. *J. gen. Microbiol.* **5**, 939.

Haynes, W. C. & Rhodes, L. J. (1962). Comparative taxonomy of crystallogenic strains of *Pseudomonas aeruginosa* and *Pseudomonas chlororaphis*. *J. Bact.* **84**, 1080.

Hugh, R. & Leifson, E. (1953). The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various Gram-negative bacteria. *J. Bact.* **66**, 24.

King, J. V., Campbell, J. J. R. & Eagles, B. A. (1948). The mineral requirements for fluorescin production. *Can. J. Res.* **26**, 514.

KING, E. O., WARD, M. K. & RANEY, D. E. (1954). Two simple media for the demonstration of pyocyanin and fluorescein. *J. lab. clin. Med.* **44**, 301.

KOVACS, N. (1956). Identification of *Pseudomonas pyocyanea* by the oxidase reaction. *Nature, Lond.* **178**, 703.

LISTON, J., WIEBE, W. & COLWELL, R. R. (1962). Preliminary studies of variations within species. *Bact. Proc.* p. 50.

LISTON, J., WIEBE, W. & COLWELL, R. R. (1963). A quantitative approach to the study of bacterial species. *J. Bact.* **85**, 1061.

LYSENKO, O. (1961). *Pseudomonas*—an attempt at a general classification. *J. gen. Microbiol.* **25**, 379.

MARTINEAU, B. & FORGET, A. (1958). Routine use of Sabouraud maltose agar for the rapid detection of the bluish-green pigment of *Pseudomonas aeruginosa*. *J. Bact.* **76**, 118.

MEADER, P. D., ROBINSON, G. H. & LEONARD, V. (1925). Pyorubin, a red water-soluble pigment characteristic of *B. pyocyaneus*. *Amer. J. Hyg.* **5**, 682.

MOLLER, F. (1962). Quantitative methods in the systematics of Actinomycetales. IV. The theory and application of a probabilistic identification key. *G. Microbiol.* **10**, 29.

PATON, A. M. (1959). Enhancement of pigment production by *Pseudomonas*. *Nature, Lond.* **184**, 1254.

PULVERER, G. & KORTH, H. (1962). Zur Biochemie und Fermentstructur von *Pseudomonas aeruginosa*. *Zbl. Bakt.* (1. *Abt. Orig.*), **186**, 36.

RHODES, M. E. (1959). The characterization of *Pseudomonas fluorescens*. *J. gen. Microbiol.* **21**, 221.

RHODES, M. E. (1961). The characterization of *Pseudomonas fluorescens* with the aid of an electronic computer. *J. gen. Microbiol.* **25**, 331.

RINGEN, L. M. & DRAKE, C. H. (1952). A study of the incidence of *Pseudomonas aeruginosa* from various natural sources. *J. Bact.* **64**, 841.

ROGERS, K. B. (1960). Pseudomonas infections in a children's hospital. *J. appl. Bact.* **23**, 533.

SELEEN, W. A. & STARK, C. N. (1943). Some characteristics of green-fluorescent pigment-producing bacteria. *J. Bact.* **46**, 491.

SHERWOOD, N. P., JOHNSON, T. L. & RADOTINCKY, I. (1926). Studies on *Bacillus pyocyaneus*. *Kans. Univ. Sci. Bull.* **15**, 91.

SHEWAN, J. M., HODGKISS, W. & LISTON, J. (1954). A method for the rapid differentiation of certain non-pathogenic, asporogenous bacilli. *Nature, Lond.* **173**, 208.

SHIMWELL, J. L., CARR, J. G. & RHODES, M. E. (1960). Differentiation of Acetomonas and *Pseudomonas*. *J. gen. Microbiol.* **23**, 283.

SIERRA, G. (1957). A simple method for the detection of lipolytic activity of micro-organisms and some observations on the influence of the contact between cells and fatty substrates. *Antonie van Leeuwenhoek*, **23**, 15.

SILVESTRI, L., TURRI, M., HILL, L. R. & GILARDI, E. (1962). A quantitative approach to the systematics of Actinomycetes based on overall similarity. *Symp. Soc. gen. Microbiol.* **12**, 333.

SNEATH, P. H. A. (1956). Cultural and biochemical characteristics of the genus *Chromobacterium*. *J. gen. Microbiol.* **15**, 70.

Society of American Bacteriologists (1957). *Manual of Microbiological Methods*. New York: McGraw-Hill Book Co., Inc.

THORNLEY, M. J. (1960). The differentiation of *Pseudomonas* from other Gram-negative bacteria on the basis of arginine metabolism. *J. appl. Bact.* **23**, 37.

VERDER, E. & EVANS, J. (1961). A proposed antigenic schema for the identification of strains of *Pseudomonas aeruginosa*. *J. infect. Dis.* **109**, 183.

WETMORE, P. W. & GOCHENOUR, W. S. (1956). Comparative studies of the genus *Malleomyces* and selected *Pseudomonas* species. *J. Bact.* **72**, 79.

WILLIAMSON, C. K. (1956). Morphological and physiological considerations of colonial variants of *Pseudomonas aeruginosa*. *J. Bact.* **71**, 617.

WOOD, A. J. & BAIRD, E. A. (1943). Reduction of trimethylamine oxide by bacteria. *J. Fish. Res. Bd Can.* **6**, 194.

J. gen. Microbiol. (1964), 37, 195–204
Printed in Great Britain

Antibacterial Action of Oxidized Spermine

BY U. BACHRACH AND S. PERSKY
Department of Clinical Microbiology, Hebrew University-Hadassah Medical School, Jerusalem, Israel

(*Received* 25 *May* 1964)

SUMMARY

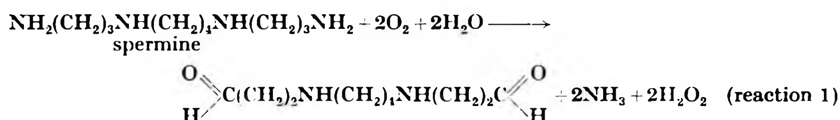
Spermine oxidized by partially purified serum amine oxidase inhibited the growth of a variety of Gram-positive and Gram-negative bacteria. This inhibition of growth was not antagonized by calcium ion, and occurred over a wide pH range. Inactivation increased with time and temperature of incubation. The antibacterial action was abolished by treating oxidized spermine with sodium borohydride. Oxidized spermine inhibited protein synthesis as shown by incorporation of ^{14}C-valine into cellular protein and by inhibition of β-galactosidase induction. Incorporation of ^{14}C-uracil into the nucleic acids of *Escherichia coli* was not inhibited by oxidized spermine.

INTRODUCTION

The naturally occurring polyamine spermine, $NH_2(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$, inhibits the growth of various bacteria, mainly Gram-positive cocci (Rozansky, Bachrach & Grossowicz, 1954). On the other hand, spermine after oxidation by crude plasma or serum amine oxidase inhibits the growth of *Mycobacterium tuberculosis* (Hirsch & Dubos, 1952; Fletcher, Epstein & Jewell, 1953). Tabor & Rosenthal (1956) studied the oxidation of spermine by purified beef plasma amine oxidase. They found that the oxidation product inhibited the growth of *Escherichia coli*, *Staphylococcus aureus* and was toxic to *Trypanosoma equiperdum* and to human spermatozoa. The inactivation of various bacteriophages by oxidized spermine was also reported (Bachrach, Tabor & Tabor, 1963).

The mechanism of spermine oxidation by purified plasma amine oxidase has recently been elucidated (Tabor, Tabor & Bachrach, 1964); this reaction proceeds as follows:

$$\underset{\text{spermine}}{NH_2(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2} + 2O_2 + 2H_2O \longrightarrow$$



$$\begin{matrix}O \\ H\end{matrix}\!\!>C(CH_2)_2NH(CH_2)_4NH(CH_2)_2C\!\!<\!\!\begin{matrix}O \\ H\end{matrix} + 2NH_3 + 2H_2O_2 \quad \text{(reaction 1)}$$

The present paper deals with the study of factors affecting the antibacterial activity of spermine oxidized by purified serum amine oxidase. It will be shown that the oxidation products interfere with the growth of various bacteria and inhibit the synthesis of some bacterial proteins. A preliminary communication on this work has appeared (Bachrach & Persky, 1963).

METHODS

Organisms. All the bacterial strains used in this work were from the collection of the Department of Clinical Microbiology, Hebrew University-Hadassah Medical School, Jerusalem.

Media. Mycobacteria were grown in the medium described by Dubos & Davis (1946). Other bacteria were grown in nutrient broth (Difco) pH 7·4.

Chemicals. Spermine tetrahydrochloride was obtained from Mann Research Laboratories, Inc. (New York, U.S.A.). Spermidine trihydrochloride was the product of California Corporation for Biochemical Research (Los Angeles 32, U.S.A.). 3,3′-Diaminodipropylamine, glyoxal, and *n*-butyraldehyde were purchased from Eastman Kodak Company. Putrescine was obtained from Nutritional Biochemicals Corporation (Cleveland, 28, Ohio, U.S.A.). Sodium borohydride was purchased from British Drug Houses, Ltd. Amine oxidase was prepared from beef serum by the method of Tabor, Tabor & Rosenthal (1954), but omitting the last calcium phosphate gel step. The preparation was purified approximately 80-fold, with a specific activity of 100 spectrophotometric units/mg. of protein.

Spermine (10 μmole) was incubated in a Warburg flask at 30° with: 100 μmole of potassium phosphate buffer (pH 7·0); 0·3 ml. (300 spectrophotometric units) of amine oxidase; 150 units of catalase (Boehringer & Soehne, Mannheim, Germany) in a final volume of 3·0 ml. The reaction went to completion in 60–120 min. with the consumption of the theoretical amount of oxygen (reaction 1 above). The incubation mixture which contained 'oxidized spermine' was used immediately or kept at −20°.

Assay. Antibacterial activity was determined by twofold dilutions in 1·0 ml. nutrient broth. To each dilution of the antibacterial agent was added 0·05 ml. of a diluted bacterial suspension containing $2\text{–}10 \times 10^6$ organisms/ml. Results were recorded after incubation at 37° for 20 hr. Viable counts were made by plating, bacterial suspensions being diluted tenfold in nutrient broth. Samples from each dilution were added to nutrient agar kept at 45°, plates poured and the number of colonies was determined after incubation at 37° for 20 hr.

Incorporation of ^{14}C-valine into bacterial protein. The incorporation of ^{14}C-valine into the protein of *Escherichia coli* was examined according to Levinthal, Keynan & Higa (1962) as follows. *E. coli* B was grown for 15–18 hr in 8 0 ml. quantities of Davis medium (Davis, 1949) supplemented with 0·5% (w/v) glucose. To each of these cultures, 10 ml. Davis glucose medium were added. The cultures were incubated for another 120 min. at 35°, and 0·33 μmole ^{14}C-valine (G) (The Radiochemical Centre, Amersham, Buckinghamshire; specific activity 2·59 μc./μmole) were then added. Samples containing 2·0 ml. of the incubation mixture were removed at various times, and added to 2·0 ml. of a solution containing 10% (w/v) trichloroacetic acid and 1% (w/v) Casamino acids (Difco Laboratories, Detroit, Michigan, U.S.A.). Samples were thoroughly shaken, kept at room temperature for 30 min. and then centrifuged at 3000*g* for 15 min. Each precipitate was then suspended in 1·5 ml. N-NaOH solution, and kept at room temperature for 30 min. Six ml. of a solution containing trichloroacetic acid and Casamino acid were then added to each suspension. The suspensions were heated in boiling water for 30 min., cooled to 20°–30° and filtered through 45 μ Millipore filters (Millipore

Filter Corp., Bedford, Mass., U.S.A.). The filters were dried, mounted on planchets and the radioactivity was determined by a Nuclear Chicago thin window gas flow counter.

Incorporation of ^{14}C-uracil into bacterial nucleic acid. Incorporation of ^{14}C-uracil into the nucleic acids of *Escherichia coli* was examined according to Levinthal *et al.* (1962). *E. coli* B was grown for 15–18 hr in 10 ml. volumes of Davis medium (Davis, 1949) supplemented with 0·5 % (w/v) glucose. To each of these cultures, 10 ml. Davis glucose medium were added. The cultures were incubated for another 120 min. at 35° and 1·6 μmole ^{14}C-uracil (Tracerlab Inc. Waltham, Mass., U.S.A., specific activity 1·04 μc./μmole) were then added. Samples containing 2·0 ml. of the incubation mixture were removed at various times and added to 2·0 ml. of a solution containing 10 % (w/v) trichloroacetic acid and 0·1 % (w/v) uracil (Light & Co. Ltd., Colnbrook, Buckinghamshire). Samples were thoroughly shaken, chilled in an ice + water bath and then filtered through 45 μ Millipore filters previously soaked in a solution containing 5 % (w/v) trichloroacetic acid and 0·05 % (w/v) uracil. The filters were then washed 5 times with 3·0 ml. quantities of 5 % trichloroacetic acid + uracil solution, dried, mounted on planchets and the radioactivity determined as above.

Induction of β-galactosidase. Escherichia coli W was inoculated into 50 ml. volumes of Davis medium (Davis, 1949) supplemented with 0·1 % (w/v) of sodium succinate. After shaking at 35° for 15 hr sodium succinate was added to a final concentration of 0·5 % (w/v) and incubation continued for another 60 min. The bacteria were harvested by centrifugation (9000*g* for 5 min.) and suspended in 12·5 ml. volumes of Davis medium supplemented with 0·5 % (w/v) sodium succinate. Six ml. of this suspension were added to about 1000 μg. of 'oxidized spermine' in 1·8 ml. of 0·06 M-sodium phosphate buffer (pH 7·0). After incubation at 35°, 0·5 ml. samples were removed at various times and added to 1·3 ml. of Davis medium + 0·5 % succinate and 0·2 ml. of a 1×10^{-2} M-solution of methyl β-D-thiogalactoside (TMG; Sigma Chemical Co. St Louis 18, Mo., U.S.A.). The samples were incubated at 35° for 10 min., then 0·2 ml. of a 0·2 % (w/v) chloramphenicol solution added. The test tubes were immediately chilled in an ice–water bath and the bacteria from each sample collected by centrifugation (9000*g* for 5 min.). To each precipitate containing induced bacteria were added 2·0 ml. of 0·05 M-sodium phosphate buffer (pH 7·5) and one drop of toluene. After incubation at 37° for 30 min., 2·0 ml. of $1·5 \times 10^{-3}$ M-*o*-nitrophenyl β-O-galactoside (ONPG) were added to each suspension. Incubation at 37° was continued for another 15 min. and the reaction was stopped by adding 2·0 ml. N-Na_2CO_3 solution to each sample. The intensity of the yellow colour formed was determined by using a Klett electrocolorimeter with a 42 filter.

RESULTS

The antibacterial activities of various naturally occurring polyamines are given in Table 1. Spermine, $NH_2(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$, was the most potent agent and inhibited the growth of *Staphylococcus aureus* 6538 at 18 μg./ml. nutrient broth. Spermidine $NH_2(CH_2)_3NH(CH_2)_4NH_2$, and $NH_2(CH_2)_3NH(CH_2)_3NH_2$, 3,3′-diaminodipropylamine were less active; 160 μg./ml. were required to inhibit growth. Putrescine, $NH_2(CH_2)_4NH_2$, was not inhibitory even at 1000 μg./ml. Spermine

oxidized by serum amine oxidase was as active as unoxidized spermine when tested against *S. aureus* at pH 7·4.

Previous experiments indicated that spermine is oxidized by serum amine oxidase to a dialdehyde (Tabor, Tabor & Bachrach, 1964). The carbonyl groups of the oxidation product seem to be responsible for the antibacterial action as indicated by the following experiment. 'Oxidized spermine' (0·6 μmole) was added to 2·0 μmole sodium borohydride and 7×10^7 *Staphylococcus aureus* organisms in 0·5 ml. nutrient broth. The number of viable bacteria was determined after 30 min. of incubation at 37°; about 90 % of the organisms were still viable. Only 0·01 % of the bacteria survived when oxidized spermine was added to the bacteria in the absence of sodium borohydride. Sodium borohydride is known to reduce carbonyl groups to the corresponding alcohols (Lyttle, Jensen & Struck, 1952).

Table 1. *Inhibition of growth of Staphylococcus aureus 6538 by various compounds*

Compounds were dissolved in 1·0 ml. volumes of nutrient broth (Difco; pH 7·4). Appropriate concentrations were prepared by twofold dilutions in nutrient broth. To each dilution were added $1-5 \times 10^5$ staphylococci. Results recorded after incubation at 37° for 20 hr.

Compound	Minimal inhibitory concentration (μg./ml.)
Spermine	18
Spermidine	160
3,3'-Diaminodipropylamine	160
Putrescine	> 1000
Oxidized spermine	18*
n-Butyraldehyde	> 5000
Glyoxal	> 500

* Based on the amount of spermine added to the original incubation mixture.

The antibacterial action of oxidized spermine was not annulled after exposing *Staphylococcus aureus* to the oxidation product for 30 min. at 37° and then adding sodium borohydride.

Other aldehydes such as *n*-butyraldehyde and glyoxal did not affect the viability of *Staphylococcus aureus* even at concentrations of 5000 and 500 μg./ml., respectively (Table 1). The antibacterial effect of oxidized spermine was not due to the serum amine oxidase preparation, which was also present in the incubation mixture. This enzyme alone, as well as catalase, had no effect on the viability of *S. aureus*.

Factors which affect the antibacterial action of oxidized spermine

Susceptibility of different bacteria. Spermine and spermidine are known to inhibit the growth of Gram-positive cocci, whereas Gram-negative bacteria are relatively resistant to their action (Rozansky *et al.* 1954). Oxidized spermine, however, inhibited the growth of the Gram-positive and the Gram-negative bacteria which were tested; the mycobacteria were also sensitive (Table 2).

Effect of pH value. The inhibition of bacterial growth by oxidized spermine was not dependent on the pH value of the culture medium. Table 3 shows that oxidized

Table 2. *Susceptibility of various bacteria to the action of oxidized spermine*

Oxidized spermine was dissolved in nutrient broth (Difco; pH 7·4). Appropriate dilutions were prepared by twofold dilutions in nutrient broth. To each dilution were added $1-5 \times 10^5$ organisms of the test organism. Results were recorded after incubation at 37° for 20 hr. Mycobacteria were grown in Dubos medium and the results were recorded after incubation at 37° for 14 days.

Organism	Minimal inhibitory concentration (μg./ml.)
Staphylococcus aureus 4996	18*
S. aureus 6538	18
Bacillus subtilis	18
Escherichia coli C	20
Proteus vulgaris	20
Salmonella paratyphi	20
Shigella flexneri	20
Pseudomonas aeruginosa	18
Serratia marcescens	18
Myobacterium tuberculosis, strain BCG	12
M. tuberculosis $H_{37}R_v$	12

* Based on the amount of spermine added to the original incubation mixture.

Table 3. *Inactivation of Staphylococcus aureus 6538 by oxidized spermine and polyamines at various pH values*

Experimental conditions as given in Table 1, except that nutrient broth at various pH values was used.

pH value	Oxidized spermine	Spermine	Spermidine	3,3′-Diamino-dipropylamine
	Minimal inhibitory concentration (μg./ml.)			
5·5	40*	·	·	·
6·0	20	·	·	·
6·5	20	75	630	630
7·4	20	18	315	315
8·3	20	2	18	18
9·0	20	·	·	·

* Based on the amount of spermine added to the original incubation mixture; · = not tested.

spermine 20 μg./ml. inhibited the growth of *Staphylococcus aureus* in a range from pH 6·0 to 9·0. The antibacterial activity of some polyamines is given for comparison. At pH 8·3 the antibacterial activity of spermine was about 40 times that observed at pH 6·5. A similar effect of pH value was noted when spermidine and 3,3′-diamino-dipropylamine were used (Table 3).

Effect of media. Staphylococcus aureus was grown in nutrient broth (Difco) at 37° for 20 hr, the cocci harvested at 4° and washed three times with cold 0·15M-NaCl. Samples of the cocci were then suspended in 0·15M-NaCl, 0·15M-NaCl + 0·05M-glucose, or nutrient broth (Difco). Oxidized spermine was added to the suspensions which were then incubated at 37° for 60 min. Samples (0·5 ml.) were taken and diluted 1/200 in cold 0·15M-NaCl and the number of viable bacteria counted by

plating. Table 4 shows that oxidized spermine was toxic to *S. aureus* suspended in 0·15 M-NaCl. Only growing multiplying *S. aureus* was affected by intact spermine, which was not toxic to washed cocci suspended in NaCl solution.

Cations such as Ca^{2+} or Mg^{2+} are known to antagonize the antibacterial action of spermine (Razin & Rozansky, 1959). The antibacterial action of oxidized spermine, on the other hand, was not antagonized by calcium ion even at 0·1 M when tested against *Staphylococcus aureus* in nutrient broth.

Table 4. *Effect of medium on the toxicity of oxidized spermine*

Staphylococcus aureus 6538 was grown in nutrient broth and washed with 0·15 M-NaCl. Cocci were then suspended in the various media containing 130 μg * of 'oxidized spermine' or 0·2 ml. of 0·15 M-sodium chloride, and incubated at 37° for 60 min.; total volume 0·4 ml. The number of viable cocci was determined by plating, the results being recorded after incubation at 37° for 20 hr.

Medium	Oxidized spermine	Number of viable cocci
Sodium chloride (0·15 M)	−	9×10^3
	+	6×10^1
Sodium chloride (0·15 M) + glucose (0·05 M)	−	2×10^3
	+	0
Nutrient broth (Difco)	−	4×10^8
	+	0

* Based on the amount of spermine added to the original incubation mixture.

Effect of temperature. Staphylococcus aureus was grown in nutrient broth at 37° for 20 hr, the cocci harvested at 4°, washed three times with cold 0·15 M-NaCl and resuspended in nutrient broth + oxidized spermine. Samples were incubated at various temperatures for 60 min. Samples (0·5 ml.) were then diluted 1/200 in cold 0·15 M-NaCl solution, and a viable count made by plating. Table 5 shows that oxidized spermine was not very toxic to *S. aureus* incubated at 4° and 25°; incubation at 30° or 37°, however, led to complete inactivation of the cocci.

Table 5. *Effect of temperature on the toxicity of oxidized spermine*

Experimental conditions were as in Table 4 except that the staphylococci were suspended in 0·15 M-NaCl. The numbers of viable cocci, after incubation for 60 min. at the temperatures indicated, were determined by plate counts, the results being read after incubation of the plates at 37° for 20 hr.

Temperature	Oxidized spermine	Control (no spermine)
	Number of viable bacteria	
4°	$3·7 \times 10^3$	5×10^3
25°	$1·8 \times 10^3$	$5·3 \times 10^3$
30°	0	$4·5 \times 10^3$
37°	0	$5·7 \times 10^2$

Effect of exposure time. Staphylococcus aureus was grown, harvested and washed as above. The washed organisms were resuspended in nutrient broth + oxidized spermine and incubated at 37°. Samples (0·5 ml.) were diluted 1/200 in cold 0·15 M-NaCl after various incubation times, and a viable count made by plating. Figure 1 shows that there was a linear relationship between the time of exposure and the log of number of survivors.

Inhibition of protein synthesis by oxidized spermine. The wide spectrum of the antibacterial activity of oxidized spermine suggested that a mechanism common to various micro-organisms is the site of the antibacterial action. The effect of oxidized spermine on the synthesis of proteins and ribonucleic acids was therefore examined. Protein synthesis was studied by (*a*) incorporation of ^{14}C-valine into trichloroacetic acid (TCA)-insoluble products; (*b*) induction of β-galactosidase.

Incorporation of ^{14}C-valine into bacterial protein. Escherichia coli B incorporated ^{14}C-valine into their protein (Fig. 2). This reaction was fast; about 50 % of the total

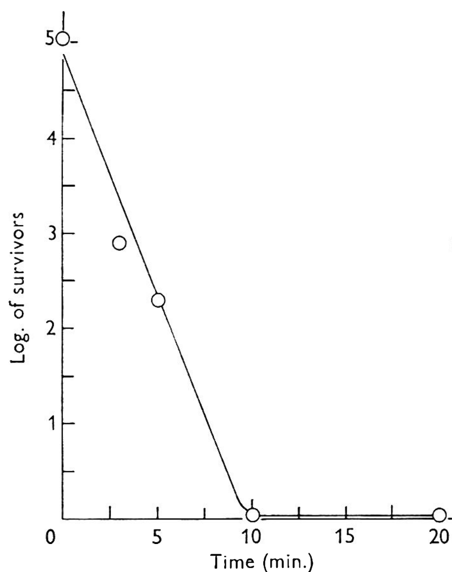


Fig. 1

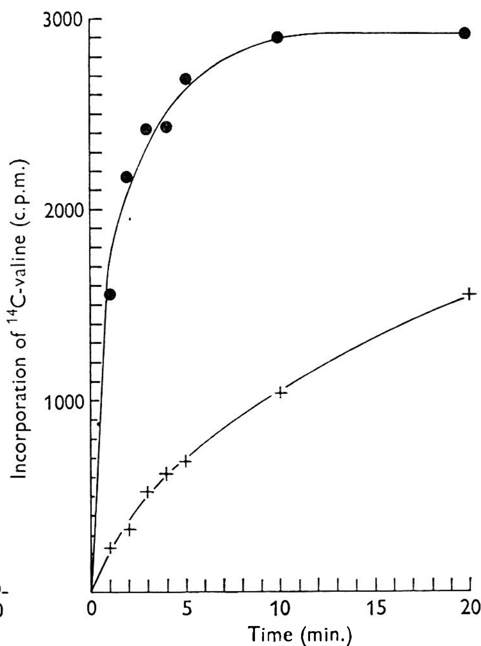


Fig. 2

Fig. 1. Effect of incubation time on the inactivation of Staphylococcus aureus by oxidized spermine.

Fig. 2. Incorporation of ^{14}C-valine into the protein of Escherichia coli B. ● — ●, incorporation system containing an equivalent of 5 mg. dry wt. bacteria/ml.; × — ×, incorporation system containing oxidized spermine 120 μg./ml. and equiv. of 5 mg. dry wt. bacteria/ml.

radioactivity was incorporated within the first minute of incubation and incorporation was complete after 10 min. A considerable inhibition of incorporation was noticed when oxidized spermine (final concentration 120 μg./ml.) was added to the bacterial suspension at zero time together with ^{14}C-valine. During the first minute of incubation, the incorporation of ^{14}C-valine into the bacterial protein was only 15 % of that obtained in the control experiment. After 2 min. of incubation, the incorporation was also about 15 % of that obtained in the control. After 20 min. of incubation, the incorporation of ^{14}C-valine in the presence of oxidized spermine was similar to that obtained in the control experiment within 1 min.

Inhibition of β-galactosidase induction. Further evidence for the effect of oxidized spermine on protein synthesis was provided by enzyme induction. Suspensions of

Escherichia coli W, in Davis medium + succinate, were incubated with oxidized spermine (final concentration 130 μg./ml.) for various periods. Methyl β-D-thiogalactoside (TMG) was then added to induce the formation of β-galactosidase. Figure 3 shows that *E. coli*, pre-incubated with oxidized spermine, was induced to a small degree only. Inhibition of induction was proportional to incubation time with oxidized spermine. When the pre-incubation was for 30 min., induction was inhibited by about 75 %; about 95 % inhibition of β-galactosidase formation was obtained with *E. coli* exposed to oxidized spermine for 90 or 120 min. Control experiments showed that treatment of *E. coli* W with oxidized spermine had no effect on the oxidation of glucose (measured by the manometric method of Warburg), neither was there any inhibition in the breakdown of β-galactosides by oxidized spermine when β-galactosidase-induced bacteria were used. These control experiments thus rule out a possible indirect effect of oxidized spermine on the metabolism of β-galactosides.

Effect of oxidized spermine on incorporation of ^{14}C-uracil into bacterial nucleic acids. Incorporation of ^{14}C-uracil into bacterial nucleic acid was studied as described in Methods. Oxidized spermine (130 μg./ml.), added to a suspension of *Escherichia coli* B and ^{14}C-uracil, had no significant effect on the incorporation (Fig. 4).

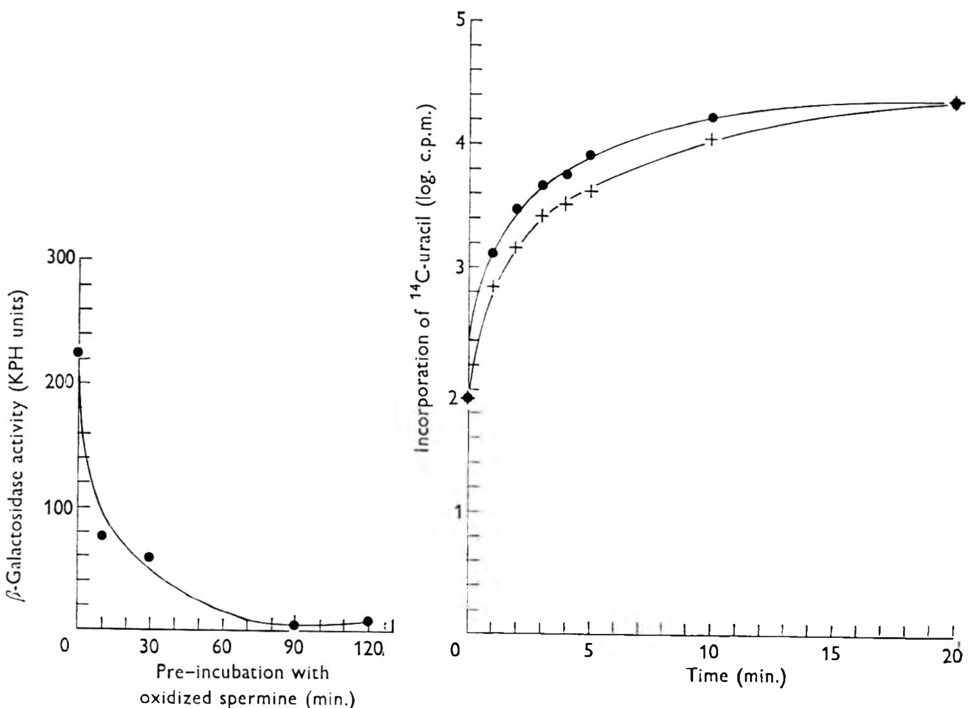


Fig. 3 Fig. 4

Fig. 3. Effect of oxidized spermine on the induction of β-galactosidase in Escherichia coli W. Bacterial suspensions (equiv. 5 mg. dry wt./ml.) were pre-incubated with oxidized spermine (130 μg./ml.) for various times. Methyl-β-D-thiogalactoside was added and β-galactosidase activity (expressed in Klett units) then determined.

Fig. 4. Incorporation of ^{14}C-uracil into the nucleic acids of Escherichia coli B. ● — ●, control system; × — ×, incorporation system containing oxidized spermine 130 μg./ml.

DISCUSSION

The antibacterial activity of the naturally occurring polyamines is apparently due to their cationic nature. These polyamines are known to form complexes with acidic components of the bacterial cell, such as DNA, RNA (Felsenfeld & Huang, 1960) or phospholipids (Razin & Rozansky, 1959), and are readily adsorbed to bacterial constituents (Bachrach & Cohen, 1961; Tabor, 1962). The antibacterial action of 'oxidized spermine' seems to be of a different nature. The carbonyl groups of the newly formed dialdehyde seem to be responsible for the toxic effect. This is borne out by the fact that sodium borohydride, which reduces the dialdehyde to the corresponding alcohol, abolished the toxic activity. Cations, such as Ca^{2+}, did not interfere with the antibacterial action of oxidized spermine, pointing to a non-electrostatic linkage between the toxic compound and the bacterial cell. The fact that oxidized spermine inhibited bacterial growth over a wide pH range also favours a non-electrostatic linkage.

The ability of oxidized spermine to inhibit the growth of a variety of bacteria is of interest, and suggests that a mechanism common to all susceptible organisms is damaged. Such a mechanism cannot involve synthesis of cell walls, which are different in the various organisms. Results presented in this paper show that oxidized spermine inhibits protein synthesis, whereas incorporation of ^{14}C-uracil into bacterial nucleic acids is unaffected. The mechanism of this inhibition is yet to be elucidated. It is possible that interaction between oxidized spermine and bacterial nucleic acids is the cause of inhibition of protein synthesis. It is reasonable to speculate that the carbonyl groups in the molecule of the oxidation product react with the amino groups of the purines and pyrimidines in the bacterial nucleic acids with the formation of Schiff bases. The formation of such compounds would not be dependent of the hydrogen ion concentration of the medium and on the presence of cations. Furthermore, such a mechanism could also explain the previously reported inactivation of various bacteriophages by oxidized spermine (Bachrach, Tabor & Tabor, 1963). We have recently found that oxidized spermine penetrates into coliphages of the T-uneven series and forms complexes with their DNA. This modified DNA is injected into the host bacterial cell after infection, but does not lead to the formation of new phages (Bachrach & Leibovici, 1964).

This work was aided by grant CA-06867 from the National Cancer Institute, United States Public Health Service.

REFERENCES

Bachrach, U. & Cohen, I. (1961). Spermidine in the bacterial cell. *J. gen. Microbiol.* **26**, 1.

Bachrach, U. & Leibovici, J. (1964). Effect of oxidized spermine on phage DNA. *Proc. 6th Int. Congr. Biochem.* **32**, 42.

Bachrach, U. & Persky, S. (1963). Effect of oxidized spermine on the incorporation of C^{14} valine into *E. coli* protein. *Israel J. Chem.* **1**, 218.

Bachrach, U., Tabor, C. W. & Tabor, H. (1963). Inactivation of bacteriophages by oxidized spermine. *Biochem. biophys. Acta*, **78**, 768.

Davis, B. D. (1949). The isolation of biochemically deficient mutants of bacteria by means of penicillin. *Proc. nat. Acad. Sci., Wash.* **35**, 1.

Dubos, R. J. & Davis, B. D. (1946). Factors affecting the growth of tubercle bacilli in liquid media. *J. exp. Med.* **83**, 409.

Felsenfeld, G. & Huang, S. (1960). The interaction of polynucleotides with metal ions, amino acids and polyamines. *Biochim. biophys. Acta*, **37**, 425.

Fletcher, F., Epstein, C. & Jewell, P. I. (1953). The antimycobacterial activity of tissue extracts and surface-active agents in Dubos's medium. *J. gen. Microbiol.* **8**, 323.

Hirsch, J. G. & Dubos, R. J. (1952). The effect of spermine on tubercle bacilli. *J. exp. Med.* **95**, 191.

Levinthal, C., Keynan, A. & Higa, A. (1962). Messenger RNA turnover and protein synthesis in *Bacillus subtilis* inhibited by Actinomycin D. *Proc. nat. Acad. Sci., Wash.* **48**, 1631.

Lyttle, D. A., Jensen, E. H. & Struck, W. A. (1952). A simple volumetric assay for sodium borohydride. *Analyt. Chem.* **24**, 1843.

Razin, S. & Rozansky, R. (1959). Mechanism of the anti-bacterial action of spermine. *Arch. Biochem.* **81**, 36.

Rozansky, R., Bachrach, U. & Grossowicz, N. (1954). Studies on the antibacterial action of spermine. *J. gen. Microbiol.* **10**, 11.

Tabor, C. W. (1962). Stabilization of protoplasts and spheroplasts by spermine and other polyamines. *J. Bact.* **83**, 1101.

Tabor, C. W., Tabor, H. & Bachrach, U. (1964). Identification of the aminoaldehydes produced by the oxidation of spermine and spermidine, with purified plasma amine oxidase. *J. biol. Chem.* **239**, 2194.

Tabor, C. W., Tabor, H. & Rosenthal, S. M. (1954). Purification of amine oxidase from beef plasma. *J. biol. Chem.* **208**, 645.

Tabor, C. W. & Rosenthal, S. M. (1956). Pharmacology of spermine and spermidine. Some effects on animals and bacteria. *J. Pharmacol.* **116**, 139.

J. gen. Microbiol. (1964), 37, 205–207
With 2 plates
Printed in Great Britain

Electron Microscope Observations of *Rhizopus rhizopodiformis*

By H. J. WERNER, P. WRIGHT and R. D. BAKER
Department of Zoology, Louisiana State University, Baton Rouge, Louisiana, U.S.A.

(*Received* 29 *May* 1964)

SUMMARY

The fine structure of *Rhizopus rhizopodiformis* was investigated. Other than a slightly modified potassium permanganate fixative, routine techniques of electron microscopy were used. The walls of the hyphae were fibrillar and electron dense. A plasmalemma, perhaps double in nature, was present. Yet to be interpreted membrane-bound vesicles were located uniformly just inside the plasmalemma. Classical ubiquitous mitochondria and smooth-surfaced endoplasmic reticulum were visible. Two inclusions, lipid and glycogen, were present. The hyphae were frequently multi-nucleated. The nuclear walls were doubled, porous, and connected with the endoplasmic reticulum.

INTRODUCTION

No account of the fine structure of *Rhizopus rhizopodiformis* has yet been published. Hawker & Abbott (1963) have made a study of the fine structure of the hyphae of *R. homothallicus* and *R. sexualis*. Several apparent differences seem to exist in these rather closely related forms and as such merit mention.

METHODS

Mycelia from a stock culture of *Rhizopus rhizopodiformis* were cultured in the following medium (g./l.): Phytone (Baltimore Biological Laboratories, Baltimore 18, Maryland, U.S.A.) 10·0, glucose 10·0, agar 15·5, cycloheximide 0·4, and chloramphemical 0·05. Cultures were incubated in the dark at 29° for 24 hr. Young hyphae were fixed in a 1 % unbuffered solution of potassium permanganate. To facilitate penetration, three drops of a 1 % solution of sodium alkane sulphonate (no. 411; Esso Research and Engineering Company, Linden, La., U.S.A.) were added to each 50 ml. of potassium permanganate. All fixed hyphae were subsequently rinsed in distilled water and then dehydrated as recommended by Spurlock, Kattine & Freeman (1963). Embedding was carried out in Maraglas (Freeman & Spurlock, 1962). Polymerization was done at 60° for 48–60 hr. Sections were cut with a DuPont diamond knife and stained with lead hydroxide as suggested by Millonig (1961). Photographs were taken with an R.C.A. EMU-3F electron microscope.

OBSERVATIONS

The cell walls of *Rhizopus rhizopodiformis* are quite similar to those of *Neurospora crassa* as described by Shatkin & Tatum (1959) and also similar to those of *R. homothallicus* as described by Hawker & Abbott (1963). The cell walls were

uniformly electron-dense structures distinctly fibrillar in nature (Pl. 1, fig. 1). A very delicate plasmalemma was detectable just inside the cell wall. There are suggestions that it was a double structure (Pl. 1, fig. 1). Of special interest were the constantly occurring, uniformly arranged, definite membrane-bound vesicles situated just inside of the plasmalemma. Occasionally, these vesicles showed additional internal organization (Pl. 1, fig. 1). With the evidence on hand, we are not in a position firmly to interpret these structures. The consensus is that these membrane-bound vesicles were a typical manifestation of pinocytosis. Despite the fact that young hyphae were used, we were never able to detect the cortical membrane described by Hawker & Abbot (1963) nor the lomosomes of Moore & McAlear (1961). We cannot confirm the observation that mitochondria display a type of polarity in that these organoids exhibit a predilection for the cortical areas of the hyphae. In *R. rhizopodiformis* the opposite prevailed—the mitochondria were ubiquitous in distribution. Mitochondria were classical in appearance with double membranes and internal cristae arranged often times in parallel plates (Pl. 1, fig. 2). The endoplasmic reticulum, devoid of attached RNA, showed smooth profiles. It ramified haphazardly throughout the cell. This system consisted principally of narrow flattened tubules and, not too infrequently, the endoplasmic reticulum assumed the form of irregular cisternae (Pl. 2, fig. 3). The contents of the cisternae were invariably less electron dense than that of the adjacent cytoplasm. Suggestions of continuity of the endoplasmic reticulum membrane were encountered (Pl. 2, fig. 4). Two inclusions were demonstrable in the cytoplasm: lipids and glycogen (Pl. 1, fig. 2; Pl. 2, figs. 3, 4). With potassium permanganate preparations, the lipids appeared as completely empty vesicles. These vesicles had characteristically distinct electron-dense membrane boundaries. Particulate clumped areas in the cytoplasm with a basophilia of varying intensity, but always greater than that of the basic granularity of the cytoplasm, were considered to be glycogen. Their size, basophilia, and general appearance agreed with the description of glycogen by Revel, Napolitano & Fawcett (1960). The hyphae of *R. rhizopodiformis* were frequently bi- and multi-nucleated (Pl. 1, figs. 1, 2). The nuclei showed considerable variation in electron density. The nuclear membrane had a typical double membrane structure. Pores were present in the wall at irregular intervals (Pl. 1. fig. 2).

This investigation was supported by Grants HE. 2549 and AI 04546–03 through the Department of Pathology, L.S.U. School of Medicine, New Orleans, La., U.S.A., and The University Council on Research.

REFERENCES

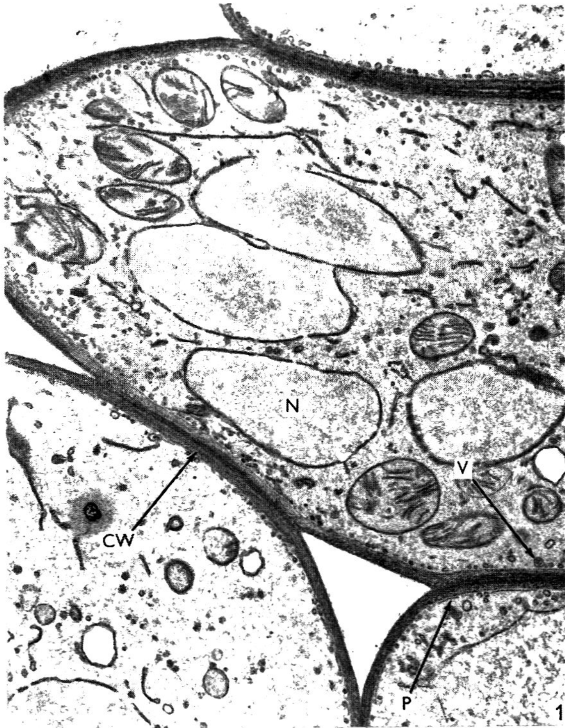
Freeman, J. A. & Spurlock, B. O. (1962). A new epoxy embedment for electron microscopy. *J. Cell Biol.* **3**, 437.

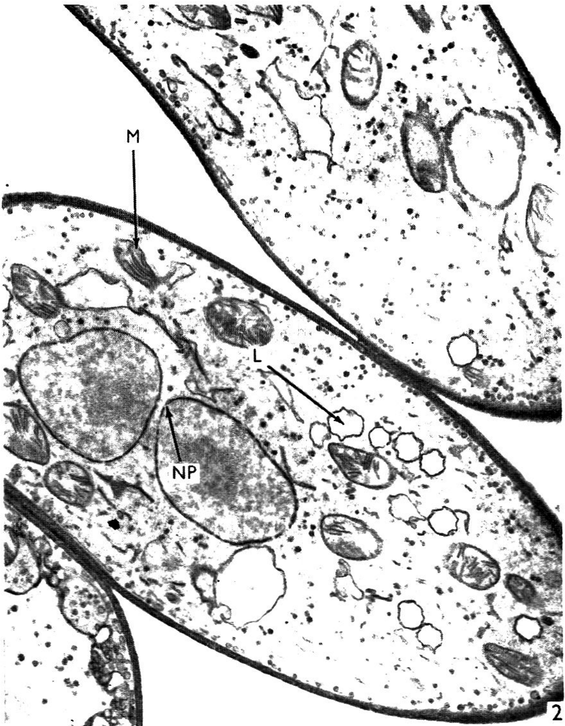
Hawker, L. E. & Abbott, P. McV. (1963). Fine structures of vegetative hyphae of Rhizopus. *J. gen. Microbiol.* **30**, 401.

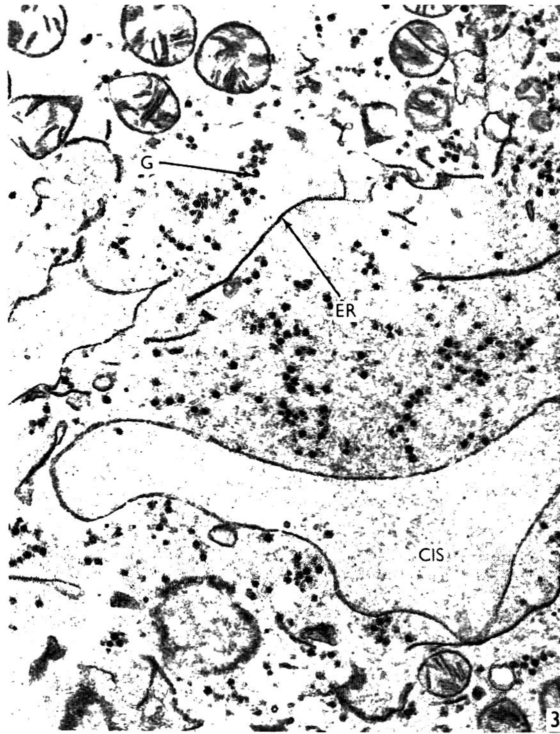
Millonig, G. (1961). A modified procedure for lead staining of thin sections. *J. biophys. biochem. Cytol.* **11**, 736.

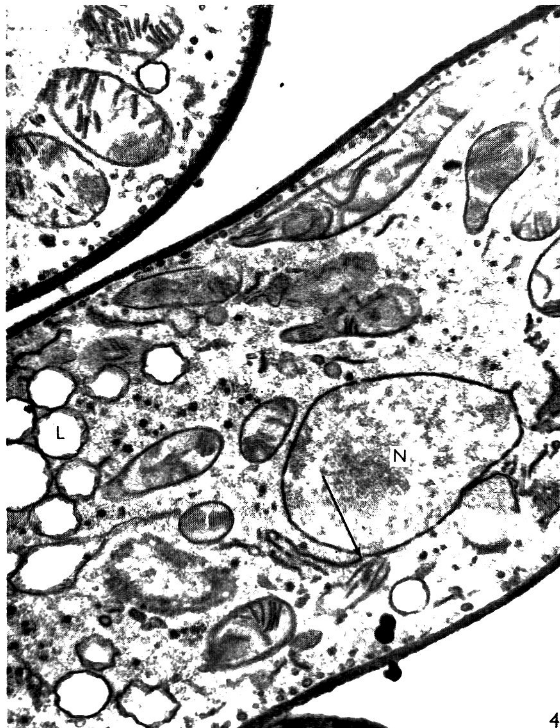
Moore, R. T. & McAlear, J. H. (1961). Fine structure of Mycota. Lomosomes—previously uncharacterized hyphal structures. *Mycologia*, **53**, 194.

Revel, J. P., Napolitano, L. & Fawcett, D. W. (1960). Identification of glycogen in electron micrographs of thin tissue sections. *J. biophys. biochem. Cytol.* **8**, 575.


N
V
CW
P
1


M
L
NP
2





H. J. WERNER, P. WRIGHT AND R. D. BAKER

SHATKIN, A. J. & TATUM, E. L. (1959). Electron microscopy of *Neurospora crassa* mycelia. *J. biophys. biochem. Cytol.* **6**, 423.

SPURLOCK, B. O., KATTINE, V. & FREEMAN, J. A. (1963). Technical modifications in Maraglas embedding. *J. Cell Biol.* **17**, 203.

EXPLANATION OF PLATES

PLATE 1

Fig. 1. Sections of *Rhizopus rhizopodiformis* showing fibrillar wall, plasmalemma, multinucleated condition, and membrane-bound vesicles. × 24,000.

Fig. 2. Section of *R. rhizopodiformis* showing nuclear pores, mitochondria with parallel cristae, and lipid inclusions. × 24,000.

PLATE 2

Fig. 3. Section of *R. rhizopodiformis* showing glycogen and the flattened and cisternal varieties of the endoplasmic reticulum. × 33,000.

Fig. 4. Section of *R. rhizopodiformis* showing a connexion of the endoplasmic reticulum with the nucleus. × 24,000.

Abbreviations

CW	cell wall	ER	endoplasmic reticulum
N	nucleus	Cis	cisternae
V	vesicle	M	mitochondrion
P	plasmalemma	L	lipid
G	glycogen	NP	nuclear pore

J. gen. Microbiol. (1964), 37, 209–223
Printed in Great Britain

The Metabolism of *Escherichia coli* and other Bacteria by *Entodinium caudatum*

BY G. S. COLEMAN

Biochemistry Department, Agricultural Research Council Institute of Animal Physiology, Babraham, Cambridge

(*Received* 2 *June* 1964)

SUMMARY

^{14}C-labelled *Escherichia coli* and other bacteria were used to measure their uptake by washed suspensions of *Entodinium caudatum*. All the bacteria tested were engulfed by the protozoa with a maximum uptake of $1{\cdot}1 \times 10^4$ *E. coli*/protozoon at an initial rate of over 200 bacteria/protozoon/min. After 30 min. only 12% of the engulfed bacteria were still viable. 50% of the bacterial carbon was retained by the protozoa and, after breakage of the protozoa and centrifugation of the homogenate, 40% of this carbon was present in the supernatant fluid, principally as protein. Competition experiments where the protozoa were offered two different species of bacteria showed that the protozoa engulfed bacteria in the proportion in which they were present in the medium. The growth of *E. caudatum* in the presence of rice starch, autoclaved rumen fluid and penicillin was stimulated by several species of bacteria, including *E. coli*, *Clostridium welchii*, *Lactobacillus casei*.

INTRODUCTION

Sheep rumen Entodiniomorphid protozoa freshly isolated from the rumen (Gutierrez & Davis, 1959) or grown *in vitro* (Coleman, 1962) contain bacteria in their gastric sacs. However, Gutierrez & Davis (1959) were able to isolate only 3–10 *Streptococcus bovis* organisms per ciliate, as compared with the 100–150 bacteria which were visible microscopically. The ciliates were also shown to ingest *S. bovis* and it was suggested that the protozoa might have been digesting the bacteria. It was found previously (Coleman, 1962) that *Entodinium caudatum* did not grow in the absence of bacteria. The present paper extends this work and by the use of ^{14}C-labelled bacteria it has been shown that many species of bacteria are engulfed and one species at least digested by the protozoa. The addition of bacteria under appropriate conditions stimulated the growth of the protozoa.

METHODS

Source of protozoa. The protozoa were grown and 'inoculum cultures' prepared and treated as described by Coleman (1962) except that 'inoculum cultures' were treated each day with 15 mg. rice starch and about 10 mg. dried grass.

Preparation of protozoa for inoculation. The protozoa were taken from the 'inoculum cultures', in which they were present as a loose pellet at the bottom of the tube, after removal of the surface scum, and most of the medium and allowed to

stand in 8 in. × 1 in. tubes until any grass present had sunk to the bottom, leaving the protozoa in the supernatant fluid. This supernatant fluid was transferred to centrifuge tubes, the residual grass washed with salt solution B (Coleman, 1960*b*) and the washings added to the supernatant fluid. The protozoa were spun down and washed three times in salt solution B which contained 0·03 % L-cysteine on a bucket-head centrifuge for 30 sec. from starting. The maximum speed was equivalent to 300**g**. The organisms were finally used as inoculum to give a population density of 5–10 × 10^4 protozoa/ml. for experiments with non-multiplying protozoa and 1–2 × 10^4/ml. for growth experiments.

Incubation conditions for engulfment of bacteria by non-multiplying protozoa. The medium consisted of 4·0–7·0 ml. (chosen so that the final volume was 10 ml.) salt solution B (Coleman, 1960*b*) autoclaved (115° for 20 min.) with 2·0 ml. water in a 15 ml. centrifuge tube. Immediately after removal from the autoclave and cooling the following additions were made aseptically: 0·2 ml. 1 % (w/v) L-cysteine hydrochloride (neutralized and Seitz filtered), 0·2 ml. 5 % (w/v) $NaHCO_3$ (Seitz filtered), 0·1–2·0 ml. suspension of ^{14}C-bacteria in salt solution B (washed once and suspended at a concentration of 10^8–10^{10} bacteria/ml.), and any other additions. After inoculation the tubes were gassed for 10 sec. with 95 % N_2 (v/v) + 5 % CO_2 (v/v), sealed with a rubber bung and incubated at 39°.

Incubation conditions for growth of protozoa. The medium consisted of 3·5–5·5 ml. (chosen so that the final volume was 10 ml.) salt solution B (Coleman, 1960*b*) autoclaved (115° for 20 min.) with 2·0 ml. water and soluble starch (if any) in a 15 ml. centrifuge tube. Immediately after removal from the autoclave and cooling the following additions were made aseptically: 0·2 ml. 1 % L-cysteine hydrochloride (neutralized and Seitz filtered); 0·2 ml. 5 % $NaHCO_3$ (Seitz filtered); 0·4 ml. penicillin G (25,000 units/ml.); 0·2 ml. 7·5 % sterile rice starch (dry rice starch heated at 120° for 24 hr. and then suspended in salt solution B), 1·0 ml. protozoa-containing autoclaved rumen fluid (PARF), 0–2·0 ml. suspension of bacteria in salt solution B (washed once and suspended at a density of 10^8–10^{10} bacteria/ml.); any other additions. After inoculation the tubes were gassed for 10 sec. with a mixture of 95 % N_2 (v/v) + 5 % CO_2 (v/v), sealed with a rubber bung and incubated horizontally at 39°. For experiments on the effect of carbohydrates, the rice starch was omitted and replaced by soluble starch which was dissolved in salt solution B and autoclaved, or by sugars which were autoclaved as 10 % solutions in water and then added to the incubation media.

At the end of experiments in which the uptake of ^{14}C-bacteria was being investigated the protozoa were centrifuged and washed twice in salt solution B on a bucket-head centrifuge for 30 sec. from starting (maximum speed was equivalent to 300**g**), and plated out to determine radioactivity. The residual bacteria were spun down from the first supernatant fluid obtained after removal of the protozoa and washed once at 2000**g** for 10 min. on an angle-head centrifuge. Samples from the supernatant fluid after removal of the bacteria were placed on planchets for estimation of the ^{14}C which was free in the medium.

Sources of bacteria used. *Bacillus megaterium* KM, *Clostridium welchii* SR 12, *Escherichia coli* B, *Leuconostoc mesenteroides* P 60 (NCIB 8952), *Nadsonia elongata*, *Salmonella typhimurium* SW 1061, *Staphylococcus aureus* (Duncan), *Streptococcus faecalis* (Dunn) and *Vibrio metchnikovii* were kindly supplied by Professor E. F.

Gale, Dr D. Kerridge and Dr K. McQuillen (of the Sub-Department of Chemical Microbiology, Department of Biochemistry, University of Cambridge). *Lactobacillus casei* var *rhamnosus* (NCIB 9282) and *Serratia marcescens* (NCIB 1377) were obtained from the National Collection of Industrial Bacteria, Torry Research Station, Aberdeen. *Streptococcus bovis* 2B (Bailey & Oxford, 1958) was obtained from the National Collection of Dairy Organisms, Shinfield, Reading (NIRD 1251). Bacterium D (a Gram-positive coccus) and Bacterium 31 (a Gram-negative rod) were isolated from standard protozoal cultures (Coleman, 1960*a*).

Bacterial growth media. Escherichia coli and *Bacillus megaterium* were grown and maintained in C medium (Roberts *et al.* 1955) containing 0·2 % glucose + 0·05 μC [U-^{14}C]-glucose/ml. (if any) at 37°. The cultures were aerated during incubation by passing sterile air into the medium through a Pasteur pipette.

Clostridium welchii, Lactobacillus casei, Leuconostoc mesenteroides, Salmonella typhimurium, Serratia marcescens, Staphylococcus aureus, Streptococcus bovis, Streptococcus faecalis, Vibrio metchnikovii and Bacteria D and 31 were grown at 37° for 16 hr in a medium that contained, per litre: salt solution (Coleman, 1958) 250 ml.; Difco yeast extract, 2 g.; Difco tryptose, 2 g.; glucose, 2 g. and [U-^{14}C]-glucose, 200 μC. (if any). All the bacteria were grown aerobically in static culture in cotton-plugged flasks except *Clostridium welchii* which was grown under CO_2.

Nadsonia elongata was grown in cotton-plugged flasks at 30° for 16 hr in a medium that contained per litre: $(NH_4)_2SO_4$, 2 g.; KH_2PO_4, 2 g.; glucose, 2g.; Difco yeast extract, 5 g.

Bacterial viable counts. Total viable counts were carried out on the medium of Bryant & Robinson (1961; hereafter referred to as Bryant medium) as described by Coleman (1962). Viable *Escherichia coli* counts were carried out by serial 100-fold dilutions in C medium + 0·2 % (w/v) glucose (Roberts *et al.* 1955) followed by plating on C medium containing 0·2 % glucose and 1 % agar and incubation at 37°. Where no *E. coli* were added to protozoal cultures the bacterial counts on this medium were less than 0·01 % of those carried out in Bryant medium.

Total bacterial counts. The total number of bacteria in a washed bacterial suspension was estimated by counting an appropriate dilution in a Helber counting chamber of depth 0·02 mm. and square size 0·0025 mm^2.

Protozoal counts. The number of protozoa was estimated by the method of Coleman (1958). Only those protozoa which showed no signs of disintegration were counted.

Rumen fluid fractions. All rumen fluid was taken from Clun Forest wethers fed on hay and oats. Protozoa-containing autoclaved rumen fluid (PARF) was prepared from fresh rumen contents by straining through muslin and autoclaving under 95 % (v/v) N_2 + 5 % (v/v) CO_2 in sealed McCartney bottles at 115° for 20 min. Rumen fluid without protozoa was prepared by straining the fluid through muslin followed by centrifugation at 500g for 3 min. to remove protozoa. This material was then used fresh or autoclaved (ARF). Autoclaved supernatant rumen fluid was prepared similarly to PARF except that the strained material was centrifuged at 12,000g for 45 min. before autoclaving. Seitz-filtered rumen fluid was prepared similarly to autoclaved supernatant rumen fluid except that sterilization was by Seitz-filtration.

Estimation of ^{14}C. ^{14}C was estimated by washing the protozoa with water on to an aluminium disc (planchet) of area 4·7 cm.^{2} carrying a disc of lens tissue. The sample

was spread by one drop of cetyltrimethylammonium bromide solution (5 mg./ml.) and fixed by one drop of polyvinyl alcohol (2 mg./ml.). The planchet was dried *in vacuo* and the ^{14}C estimated by using a thin mica end-window GM tube and conventional scaler equipment. Over 1000 counts at a rate greater than five times background were recorded for all fractions. Most determinations were carried out with less than 0·5 mg. material/cm.^{2} of planchet. Where more material was present, the results were corrected to infinite thinness by using corrections determined from known weights of the appropriate pure organic compounds, mineral salts or rumen fluid fractions added to tracer quantities of ^{14}C compounds. To determine the relative ^{14}C contents of spots on a chromatogram a thin mica end-window GM tube was placed directly on the spot as determined by radioautography.

Fractionation of protozoa. The protozoa were first broken by treatment in an all-glass Potter homogenizer (Potter & Elvehjem, 1936) at room temperature until 98–100 % of the organisms were broken (usually about 90 sec.) and then the homogenate was made up to a known volume and centrifuged at 7000*g* for 20 min. The supernatant fluid from this centrifugation is hereafter referred to as the 'broken-cell supernatant fluid' and the pellet after washing once in water as the 'broken-cell pellet'. This latter fraction contained all the viable bacteria in the homogenate.

These two fractions were further fractionated by a method based on that of Schneider (1945) and Roberts *et al.* (1955). The fraction was treated with 5 % (w/v) trichloroacetic acid (TCA) and allowed to stand at 4° for 30 min. The precipitate was centrifuged down and washed once in 5 % TCA. The supernatant fluid and the washings formed the 'cold TCA soluble fraction'. The precipitate was then extracted with 5 % TCA at 100° for 30 min. The residue was centrifuged down and washed once with 5 % TCA. The supernatant fluid + washings formed the 'hot TCA soluble or nucleic acid fraction'. The TCA was removed from both fractions by washing three times with ether before placing samples on planchets for the estimation of ^{14}C. The residue after further washing, once with ethanol acidified with 0·01 N-HCl and once with ether, formed the protein fraction. This protein was hydrolysed completely by heating at 105° in 6 N-HCl for 16 hr in a sealed tube. The tube was then cooled, opened and the acid removed on a boiling water bath in a current of air. The amino acids were separated by two-dimensional paper chromatography in *sec*-butanol + formic acid + water (70 + 10 + 20, by vol.) and phenol + ammonia (sp.gr. 0·880) + water (80 g. + 0·3 ml. + 20 ml.). ^{14}C-Amino acids were detected by radioautography.

Chemicals. ^{14}C-compounds were supplied by the Radiochemical Centre, Amersham, Buckinghamshire. The specific activities of the compounds as supplied were, in μC./mg.: [U-^{14}C]-glucose 47·7; [8-^{14}C]-guanine 70·7; [U-^{14}C]-L-leucine 320.

RESULTS

The metabolism of Escherichia coli by non-multiplying Entodinium caudatum

The method used to study the uptake of bacteria by *Entodinium caudatum* was to measure the incorporation by the protozoa of ^{14}C from ^{14}C-labelled bacteria of known specific activity. For most of this work the bacterium used was *Escherichia coli* B because: (*a*) it could be prepared uniformly labelled with ^{14}C by growth on [U-^{14}C]-

glucose as sole source of carbon; (*b*) it grew aerobically on plates on glucose ammonia salts agar medium under which conditions less than 0·1 % of the bacteria which grew from the protozoal inoculum during 24 hr in the protozoal growth medium formed colonies. It was therefore possible to measure the incorporation of bacterial carbon into the protozoa and the disappearance of viable *E. coli* from the medium. The bacteria were separated from the protozoa by differential centrifugation as described under 'Methods'. Table 1 shows that under the conditions used

Table 1. *Effect of various incubation conditions on the engulfment of ^{14}C-Escherichia coli by Entodinium caudatum*

Experiment carried out with $1 \cdot 3 \times 10^9$ Escherichia coli organisms/ml. (3710 counts/min.) in the presence or absence of $6 \cdot 2 \times 10^4$ protozoa/ml. at the temperature indicated. The 'protozoal fraction' was the pellet (if any) obtained by centrifugation for 30 sec. from starting at a maximum of 300 *g* and washing twice in salt solution B under the same conditions.

Time (hr)	Conditions	^{14}C found in 'protozoal fraction' (counts/min.)
0	Bacteria only	12
3	Bacteria only, 39°	24
0	Bacteria + protozoa	5
3	Bacteria + protozoa, 39°	368
3	Bacteria + protozoa, 4°	3
3	Bacteria + broken protozoa*, 39°	10
3	Bacteria + protozoa boiled for 5 min., 39°	15

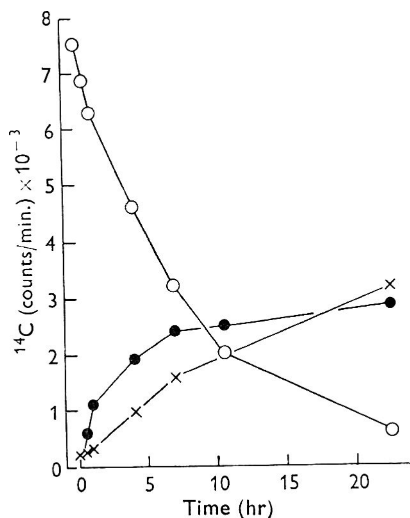
* Protozoa broken in a Potter homogenizer.

there was little incorporation of ^{14}C from ^{14}C-*E. coli* into the 'protozoal fraction' initially, in the presence of broken or boiled protozoa, after incubation at 4°, or in the absence of protozoa. The incorporation as measured over a 3-hr period had a temperature optimum at 35–40° and the rate dropped to less than 5 % of the optimum at 21° and 45°. The ^{14}C incorporation was also sensitive to decreases in the salt concentration of the medium. At a salt concentration 25 % of normal the incorporation as measured over 2 hr decreased to 10 % as did the number of protozoal survivors at the end of the experiment. These results indicate that incorporation of ^{14}C from ^{14}C-labelled *E. coli* into the protozoal fraction occurred only in the presence of intact metabolizing protozoa. Although the same result might have been obtained if the protozoa caused extensive clumping of the bacteria, no large clumps of bacteria were visible in washed protozoal suspensions at the end of experiments. It is therefore suggested that the bacteria were engulfed by the protozoa.

Attempts to recover viable Escherichia coli inside protozoa. To determine whether the bacteria engulfed by the protozoa were still viable the following experiment was carried out. During 5 hr, $8 \cdot 4 \times 10^4$ protozoa/ml. were allowed to engulf ^{14}C-labelled *Escherichia coli* from a suspension that contained 65×10^7 bacteria/ml. At the beginning and at intervals during the experiment the ^{14}C was determined in the protozoa and the viable bacteria in the medium, in washed intact protozoa and in washed protozoa broken in a Potter homogenizer. At 30 min. and 5 hr, respectively,

each protozoon contained ^{14}C from 770 and 1520 bacteria (as determined from ^{14}C in the protozoa) but there were only 95 and 15 viable bacteria inside each protozoon (as determined from the difference in viable count between intact and broken protozoa). These results show that *E. coli* was rapidly rendered non-viable after engulfment by the protozoa.

Time course of metabolism of Escherichia coli. This is shown in Fig. 1. After 22 hr incubation of protozoa with ^{14}C-labelled *Escherichia coli*, over 90 % of the free bacteria had disappeared (as determined by ^{14}C estimations and bacterial viable counts) and approximately half the bacterial carbon then present was found in the protozoa and half in the supernatant fluid.



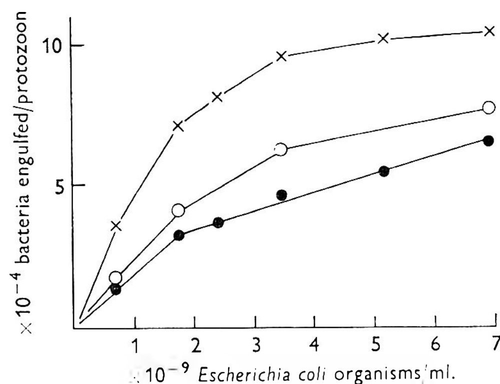


Fig. 1 Fig. 2

Fig. 1. Metabolism of [U-^{14}C]-Escherichia coli ($9{\cdot}0 \times 10^8$ organisms/ml.; 7550 counts/min./ml.) by $7{\cdot}3 \times 10^4$ Entodinium caudatum/ml. ●——● ^{14}C in protozoa; ○——○ ^{14}C in free bacteria; ×——× ^{14}C free in medium.

Fig. 2. Effect of Escherichia coli population density on engulfment by $7{\cdot}2 \times 10^4$ Entodinium caudatum/ml. in the presence and absence of 10 % supernatant fluid from centrifugation of PARF at 7000 *g* for 20 min. ●——● incubation for 30 min. without PARF supernatant; ○——○ incubation for 30 min. with PARF supernatant; ×——× incubation for 3 hr with or without PARF supernatant.

Effect of bacterial concentration on the uptake of Escherichia coli. The uptake of bacteria increased with increasing initial concentration of bacteria (Fig. 2). After 30 min. at an initial bacterial concentration of $7{\cdot}0 \times 10^9$ *Escherichia coli*/ml. in the presence of $7{\cdot}2 \times 10^4$ protozoa/ml. the uptake was $6{\cdot}5 \times 10^3$ bacteria/protozoon; this was increased to $7{\cdot}9 \times 10^3$ bacteria/protozoon in the presence of 10 % PARF (clarified by centrifugation). To determine the maximum uptake of bacteria the reciprocal of the bacterial uptake at 30 min. and 3 hr was plotted against the reciprocal of the bacterial concentration and the graphs extrapolated to infinite bacterial concentration. The correction was a small one for the 3-hr results and at both times the maximum uptake of *E. coli* was $1{\cdot}1 \times 10^4$ bacteria/protozoon. Although 10 % clarified PARF stimulated the rate of uptake of *E. coli*, 10 %

PARF (as prepared) and which contained dead bacteria, decreased by 30 % the uptake of *E. coli* from a suspension of $1 \cdot 3 \times 10^9$ bacteria/ml. In eight experiments on the engulfment of *E. coli* the highest value of the maximum uptake was 11×10^3, the lowest 4×10^3 and the average $6 \cdot 3 \times 10^3$ *E. coli*/protozoon. The actual value may depend on the volume of the gastric sac occupied by starch grains on harvesting the protozoa, since a large number of starch grains might be expected to decrease the volume available for the engulfment of bacteria.

The volume occupied by each *Escherichia coli* organism when closely packed was determined as 2 μ^3, by centrifugation of a suspension containing a known number of bacteria for 10 min. at 3000*g* and measuring the volume of the cell pad. The maximum and minimum gastric sac volumes as determined from the number of bacteria and the volume of each bacterium were therefore $2 \cdot 2 \times 10^4$ and $8 \cdot 0 \times 10^3$ μ^3, respectively. At a suspension concentration of $6 \cdot 5 \times 10^8$ *E. coli* organisms/ml. there was one bacterium per $1 \cdot 54 \times 10^3$ μ^3 medium. At this bacterial concentration after 30 min. in the presence of PARF supernatant fluid, $1 \cdot 7 \times 10^3$ bacteria/protozoon had been engulfed (Fig. 2). Therefore the bacteria from $2 \cdot 6 \times 10^6$ μ^3 medium had been removed by each protozoon. If it be assumed that there is a continuous passage of medium through a protozoon from mouth to anus, and that any bacteria in this medium are filtered off and remain in the protozoon, and if A = 'volume of medium which contained the bacteria engulfed by each protozoon', B = 'gastric sac volume' and C = 'number of gastric sac volumes of medium passing through the protozoon', then $A/B = C$. Therefore in 30 min. $2 \cdot 6 \times 10^6 \div 2 \cdot 2 \times 10^4 = 120$ gastric sac volumes of medium have passed through each protozoon. Since the most rapid uptake of bacteria occurred during the first 30 min. of incubation, these volumes are maximum values; it is possible that when the protozoa withdraw their cilia and become quiescent no medium passes through them.

Uptake of various particulate fractions prepared from ^{14}C *Escherichia coli.* Live bacteria, bacteria that had been boiled for 5 min. and then washed, bacteria treated with 5 % trichloroacetic acid and then washed and bacterial protein prepared by the method of Roberts *et al.* (1955) and then broken-up in a Potter homogenizer, were all taken up by protozoa and digested to about the same extent when suspended in equivalent amounts. In contrast, the uptake of ^{14}C from the hot trichloroacetic acid extract of the bacteria (after removal of the trichloroacetic acid with ether) was less than 3 % of that of any particulate preparation.

Table 2 shows that in an experiment where all the protein (prepared from *Escherichia coli* grown on [U-^{14}C]-glucose) was engulfed in the first 30 min. there was subsequently an increase in the ^{14}C in the protein of the broken-cell supernatant fluid and the medium, whereas the ^{14}C in the broken-cell pellet protein and cell 'pool' diminished. This result is consistent with an engulfment of the protein particles (which in the undegraded form would appear in the broken-cell pellet fraction) into the gastric sac, followed by digestion to small molecular-weight compounds such as amino acids, which were then released into the medium or incorporated into protozoal soluble protein.

Metabolism of ^{14}C*-leucine and* ^{14}C*-guanine labelled Escherichia coli.* In an attempt to study the metabolism of protein and nucleic acid of living bacteria by protozoa, *Escherichia coli* was grown on C medium containing ^{12}C-glucose and either [U-^{14}C]-L-leucine or [8-^{14}C]-guanine. Roberts *et al.* (1955) found, and it was confirmed in

the present work, that growth in the presence of [U-^{14}C]-L-leucine produced bacteria labelled only in the leucine of the bacterial protein, and that growth in the presence of ^{14}C-guanine produced bacteria labelled in nucleic acid, guanine and to a lesser extent adenine.

Table 2. *The metabolism of Escherichia coli protein by Entodinium caudatum*

Protozoa (Entodinium caudatum) incubated anaerobically in the presence of Escherichia coli protein (8800 counts/min.) prepared from E. coli grown on [U-^{14}C]-glucose by the method of Roberts *et al.* (1955). The results are expressed in counts/min. After incubation the washed protozoa were broken in a Potter homogenizer and the supernatant and pellet fractions separated by centrifugation.

Time (hr)	Protozoa present	^{14}C in 'protozoal fraction'	^{14}C in medium	Cold trichloroacetic acid soluble fraction	Nucleic acid	Protein
		Radioactivity (counts/min.)				
0	±	732	200	—	—	—
0·5	+	6430	1580	—	—	—
		Broken cell supernatant	—	2130	52	350
		Broken cell pellet	—	105	150	3650
5·5	+	3590	3630	—	—	—
		Broken cell supernatant	—	400	55	925
		Broken cell pellet	—	110	190	1910
5·5	—	240	1000	—	—	—

The course of the digestion of ^{14}C-leucine labelled *Escherichia coli* was similar to that obtained with bacteria labelled by growth in the presence of [U-^{14}C]-glucose (Fig. 1). Of the ^{14}C retained by the protozoa approximately 40 % was in the broken-cell supernatant fraction, and over 90 % of the ^{14}C in the supernatant fluid and pellet fractions was present as protein-leucine. In the absence of protozoa less than 5 % of the bacterial ^{14}C was liberated into the medium in 60 hr. The presence of 0·01 M-^{12}C-L-leucine in the medium during the experiment decreased the incorporation of ^{14}C into the protozoa by only 20 % in the first hour, but thereafter the ^{14}C in the protozoa remained constant, while in the absence of ^{12}C-leucine it steadily increased. It was not possible to abolish completely the incorporation of ^{14}C by the addition of increased amounts of ^{12}C-leucine. The lowest concentration that gave maximal effect was 0·005 M and there was no increased effect when this was increased to 0·02 M.

The metabolism of ^{14}C-guanine-labelled *Escherichia coli* differed from that of ^{14}C-leucine labelled *E. coli* in that after incubation for 27 hr, when 98 % of the bacteria had been engulfed (i) over 70 % of the ^{14}C was present in the medium, and (ii) 75 % of the protozoal ^{14}C was present in the broken-cell supernatant fluid, and only 30 % of this was in the nucleic acid, the remainder being in the cold trichloroacetic acid soluble material. However, these results were difficult to interpret because in the absence of protozoa 25 % of the bacterial ^{14}C was liberated into the

medium. The nucleic acid ^{14}C was present only in the adenine and guanine which were labelled in the same proportions as in the engulfed bacteria.

The engulfment of other micro-organisms by non-multiplying protozoa

The method used to study the engulfment of other bacteria and a yeast was similar to that used for *Escherichia coli* except that the micro-organisms were grown on complex organic materials + [U-^{14}C]-glucose and were therefore probably not uniformly labelled with ^{14}C. When the organisms were incubated for 3 hr at an organism/protozoon ratio of 50–500, 21–45 % of a suspension of the following micro-organisms was taken up by the protozoa: *Bacillus megaterium* KM, *Clostridium welchii* SR 12, *Leuconostoc mesenteroides*, *Nadsonia elongata* (a yeast), *Salmonella typhimurium* SW 1061, *Serratia marcescens*, *Streptococcus bovis* 2B, *Streptococcus faecalis* (Dunn), *Vibrio metchnikovii*, Bacterium D, Bacterium 31. All the organisms tested were engulfed by the protozoa.

Competition between bacteria for engulfment by Entodinium caudatum. In an attempt to determine whether the protozoa engulfed some bacteria preferentially, a washed suspension of protozoa was incubated for 3 hr in the presence of a concentration of ^{14}C-*Escherichia coli* such that any further increase in the concentration of bacteria caused negligible increase in the number of bacteria taken up per protozoon, i.e. almost a saturating concentration. The effect on uptake of ^{14}C-*E. coli* of adding varying volumes of suspensions of ^{12}C-bacteria, containing a known number of bacteria, to a fixed saturating concentration of *E. coli* suspension, was then studied. The volume required to decrease the uptake of ^{14}C into the protozoa to 50 % of that in the absence of ^{12}C-bacteria was determined. From the relative numbers of *E. coli* and other bacteria present the number of *E. coli* equivalent to each of the other bacteria under these conditions was determined. For example, Fig. 3 shows an experiment in which ^{12}C-*E. coli* and ^{12}C-*Lactobacillus casei* were in competition with 25×10^8 ^{14}C-*E. coli*/ml. The number of these bacteria required to decrease the engulfment of ^{14}C-*E. coli* to 50 % was 24×10^8 and $8{\cdot}5 \times 10^8$/ml. respectively. Therefore each ^{12}C-*E. coli* and ^{12}C-*L. casei* was equivalent to 1·04 and 2·9 ^{14}C-*E. coli*, respectively. It is of interest that each *L. casei* occupied 2·4 times as much space as *E. coli* as determined from the packed volumes of the bacteria. The following list gives, first the number of *E. coli* equivalent to each bacterium as determined under these conditions, and second the volume occupied by each bacterium relative to that of *E. coli*: *Bacillus megaterium* KM, 27, 24; *Clostridium welchii* SR 12, 4, 5; *Lactobacillus casei*, 2·9, 2·4; *Nadsonia elongata*, 22, 21; *Staphylococcus aureus* (Duncan), 2, 1·3; *Streptococcus bovis* 2B, 2, 1·4; *S. faecalis* (Dunn), 0·6, 0·72; Bacterium D, 1·1, 0·9; Bacterium 31, 2·2, 1·8. These results suggest that the efficiency of any bacterium in diminishing the engulfment of ^{14}C-*Escherichia coli* was approximately proportional to its size.

Growth of Entodinium caudatum in the presence of Escherichia coli

It was reported previously (Coleman, 1960*b*) that in the presence of rice starch, dried grass, autoclaved rumen fluid (PARF) and penicillin, *Entodinium caudatum* which had been maintained *in vitro* culture for 18 months grew for 1–2 generations in 4–7 days. However, after 3 years of maintenance *in vitro* maximal growth of the protozoa only occurred when living bacteria were added to the culture; there was no

stimulation in the presence of boiled bacteria. This effect was not specific to one bacterial preparation and was found with crude rumen contents from which protozoa had been removed by centrifugation (Fig. 4), and with *Bacillus megaterium* KM, *Escherichia coli* B (Fig. 4), *Clostridium welchii* SR12 (Fig. 4), *Nadsonia elongata, Lactobacillus casei, Streptococcus faecalis* (Dunn) and *S. bovis* 2B at population densities of 10–100 × 10^7 organisms/ml. In contrast, Bacterium 31 (an unidentified Gram-negative rod) which was isolated from a protozoal culture and which produced slime on addition to incubation media, inhibited protozoal growth. Growth in the

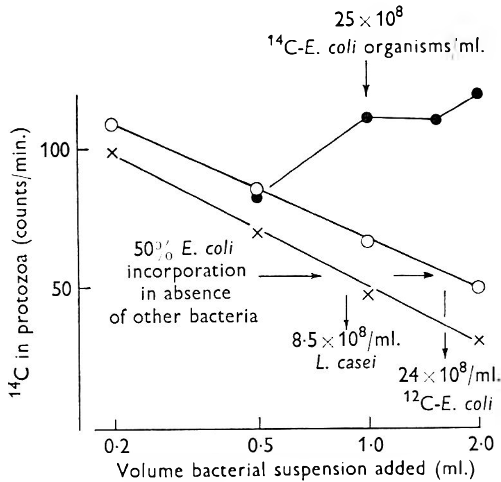


Fig. 3

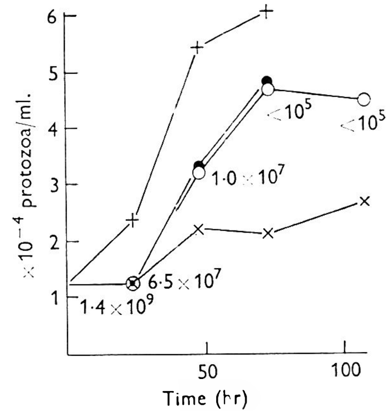


Fig. 4

Fig. 3. Effect of population density of ^{12}C-Escherichia coli and ^{12}C-Lactobacillus casei on the engulfment of [U-^{14}C]-Escherichia coli by 3·8 × 10^4 Entodinium caudatum/ml. after 3 hr. The total volume was 10 ml. ●——●, effect of ^{14}C-Escherichia coli (25 × 10^9/ml. and 1510 counts/min./ml.) population density on ^{14}C incorporated by protozoa; ○——○ effect of ^{12}C-E. coli (14 × 10^9/ml.) population density on incorporation of ^{14}C from 1·0 ml. ^{14}C-E. coli; ×——×, effect of ^{12}C-Lactobacillus casei (9·4 × 10^9/ml.) on incorporation of ^{14}C from 1·0 ml. ^{14}C-Escherichia coli.

Fig. 4. Effect of various bacterial preparations on the growth of Entodinium caudatum under standard conditions. No added bacteria ×——×; 5 % crude rumen contents (after removal of protozoa) +——+; 3·5 × 10^8 Clostridium welchii/ml. ●——●; 1·4 × 10^9 Escherichia coli/ml. ○——○ (the figures indicate the number of viable E. coli at each point).

presence of stimulatory bacteria was decreased by 25 % when tubes were incubated vertically instead of horizontally. As the protozoa in tubes that were incubated vertically contained less starch (as determined microscopically after staining with iodine) this result may be associated with the inability of protozoa to obtain sufficient starch for maximal growth when the starch is as a pellet at the bottom of a tube. During growth in the presence of *E. coli* the number of viable *E. coli* organisms in the medium decreased rapidly until after 72 hr less than 0·01 % of the original number remained (Fig. 4). In the absence of protozoa the number of viable *E. coli* organisms decreased by 25 and 75 % in 48 hr and 96 hr, respectively.

Growth of other bacteria during growth of protozoa in presence or absence of Escherichia coli. The number of other bacteria as determined by viable counts in Bryant

medium increased from 1–3 × 10^5 to about 50 × 10^7/ml. over the first 36 hr in the presence or absence of *Escherichia coli* and usually remained constant thereafter for more than 60 hr. However, in some experiments the numbers decreased to 1–10 × 10^7/ml. after 48 hr and then stayed constant or increased slowly to 50 × 10^7/ml.

Effect of Escherichia coli concentration on protozoal growth. Fig. 5 shows that the lowest initial population density of *Escherichia coli* that produced the maximum growth stimulation was 4 × 10^4 bacteria/protozoon inoculated, and that higher bacterial concentrations did not increase the growth rate further, although at the highest concentration tested it was occasionally depressed.

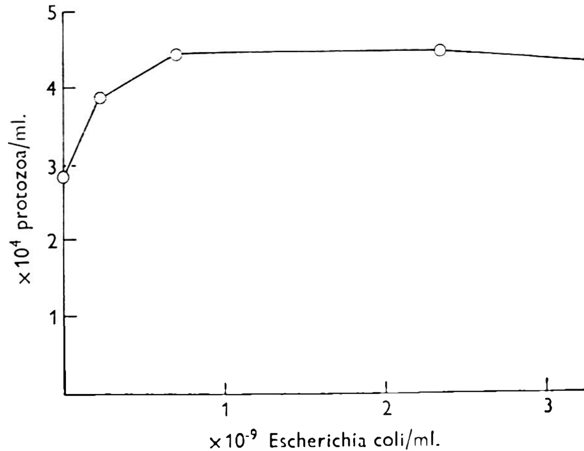


Fig. 5. Effect of initial population density of Escherichia coli on growth of Entodinium caudatum from a 1·8 × 10^4/ml. inoculum after 50 hr.

Effect of autoclaved rumen fluid fractions on protozoal growth in presence of Escherichia coli. There was no increase in the number of protozoa after 48 hr in the absence of protozoa-containing autoclaved rumen fluid (PARF) and 2 and 5% PARF gave 28 and 75%, respectively, of the growth obtained with 10% PARF. Higher concentrations of PARF were inhibitory. Autoclaved rumen fluid supernatant and Seitz filtered rumen fluid supported no growth, and ARF had only 60% of the activity of PARF. To determine whether the soluble or insoluble materials were important PARF was fractionated by centrifugation into a clear supernatant fluid and a pellet fraction which was washed free from supernatant fluid. When tested at 10% in the presence of bacteria the supernatant fluid fraction contained 0–30% of the growth-promoting activity in PARF and the number of protozoa never doubled, however long the incubation period. In contrast, the pellet fraction contained 60–70% of the total activity and the number of protozoa usually doubled in 60 hr. In the absence of bacteria the number of protozoa declined in the presence of both fractions. These results suggest that particulate matter, which was presumably engulfed by the protozoa, was more important for growth under these conditions than were soluble materials.

Replacement of starch grains by other carbohydrates. Of the carbohydrates tested, growth comparable to that in the presence of starch grains was found only with soluble starch and maltose (Fig. 6). Replacement of the starch grains by 1% cellobiose, glucose or sucrose produced approximately half maximum growth and

there was no growth during 72 hr in the presence of a 1 % solution of arabinose, fructose, galactose, inulin, mannose, melibiose or xylose, although in their absence the protozoa died in 48 hr. The growth of bacteria other than *Escherichia coli*, as determined by viable counts in Bryant medium, in the presence of soluble starch or maltose was usually the same as, and never more than double, that in the presence of starch grains.

Metabolism of uniformly labelled ^{14}C-Escherichia coli. During growth of protozoa in the presence of uniformly labelled ^{14}C-*Escherichia coli* about 30 % of the bacterial-carbon was assimilated by the protozoa and the remainder appeared in the medium.

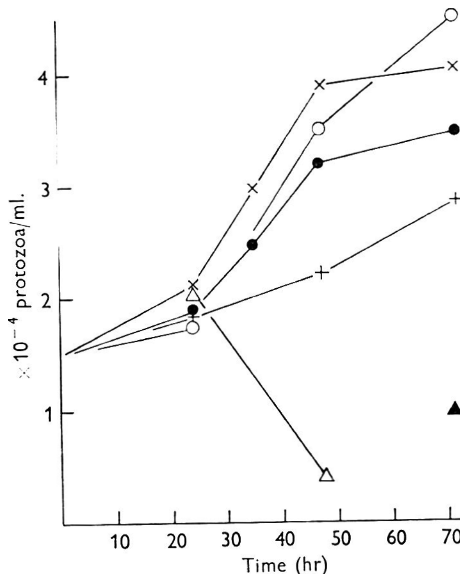


Fig. 6

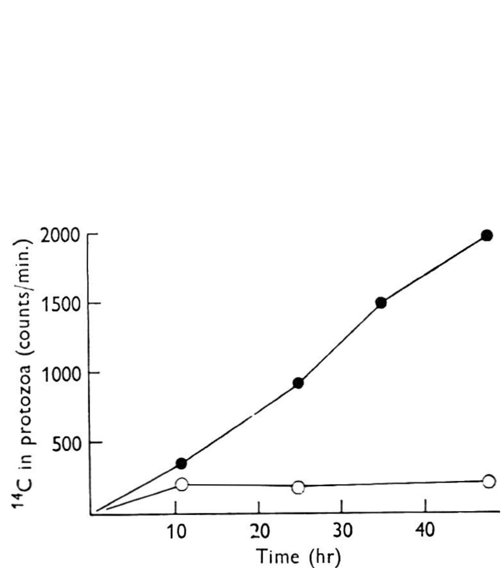


Fig. 7

Fig. 6. Effect of carbohydrates on the growth of Entodinium caudatum in the presence of $2 \cdot 2 \times 10^9$ Escherichia coli/ml. under standard conditions except that rice starch was omitted or replaced by other carbohydrates. △——△, no addition; ×——×, +0·15 % rice starch grains; ●——●, +0·6 % soluble starch; ○——○, +1 % maltose; +——+, +1 % glucose; ▲, +1 % fructose.

Fig. 7. Effect of 0·01 M-^{12}C-L-leucine on the incorporation of ^{14}C from ^{14}C-leucine labelled Escherichia coli during growth of Entodinium caudatum. ●——● no added ^{12}C-L-leucine; ○——○ +0·01 M-^{12}C-L-leucine. $1 \cdot 1 \times 10^4$ protozoa/ml. and $1 \cdot 7 \times 10^9$ E. coli/ml. (8300 counts/min./ml.) present initially.

After breakage of these protozoa in a Potter homogenizer 45 % of the ^{14}C was in the broken cell supernatant fluid and 70 % of this was present in the protein fraction. In the absence of protozoa only 10–13 % of the bacterial-carbcn was liberated into the medium over 48 hr.

Metabolism of ^{14}C-leucine labelled Escherichia coli. In an attempt to follow the metabolism of *Escherichia coli* protein during protozoal growth, the bacteria were labelled with [U-^{14}C]-leucine and the incorporation of ^{14}C from these bacteria was compared with the uptake of ^{14}C from [U-^{14}C]-L-leucine free in the medium in the presence and absence of ^{12}C-*E. coli*. In the experiment shown in Fig. 7, the number of protozoa increased by 140 % in the presence of, and 35 % in the absence of *E. coli*

during 48 hr.; 24 % of the ^{14}C from the ^{14}C-*E. coli* was found in the protozoa and the remainder in the medium. Over 95 % of the protozoal ^{14}C was in the protein fraction as leucine. In the absence of protozoa the amount of ^{14}C that appeared in the medium was decreased by 85 %. The addition of 0·01 M-^{12}C-L-leucine to the incubation medium diminished the incorporation into the protozoa to 3 %, suggesting that, as with non-multiplying protozoa, the leucine in the bacterial protein was hydrolysed in the gastric sac to the free amino acid before incorporation into protozoal protein. From the results of Roberts *et al.* (1955) on the composition of *E. coli* it was calculated that the $1·7 \times 10^9$ bacteria/ml. present initially in this experiment contained 23 μg. leucine of which 5·5 μg. (24 %) was incorporated into the protozoa. In contrast only 4 % of the ^{14}C from 0·013 μg. [U-^{14}C]-L-leucine/ml. and less than 1 % from 13 μg. leucine/ml. free in the medium was taken up by the protozoa. In the presence of ^{12}C-*E. coli* the uptake of ^{14}C-leucine was increased, probably due to incorporation after assimilation by the bacteria. These results show that leucine was utilized much more economically from intact bacterial protein which was broken down in the gastric sac than from the free amino acid which could probably only be taken up during passage of the medium through the gastric sac.

Attempts to replace living bacteria for protozoal growth

Since protozoa can engulf living bacteria, dead bacteria and particles of bacterial protein, the necessity for living bacteria for growth must be associated with some other property, possibly the accelerated reduction in the redox potential of the medium. To test this possibility tubes of medium with soluble starch as source of carbohydrate were pre-incubated anaerobically for 16 hr in the presence and absence of 4×10^8 *Escherichia coli*/ml. Two of the tubes that contained *E. coli* were then heated at 80° for 5 min. to kill the bacteria. 4×10^8 *E. coli*/ml. was then added to one of the heated tubes and also to one of the tubes pre-incubated in the absence of *E. coli*. At the same time fresh tubes of medium with soluble starch as source of carbohydrate were set up in the presence and absence of 4×10^8 *E. coli*/ml. and all the tubes inoculated with $2·3 \times 10^4$ protozoa/ml. These tubes were gassed with a mixture of 95 % (v/v) N_2 + 5 % (v/v) CO_2 and sealed. After 24 hr the number of protozoa had increased by 45 % in all tubes that on protozoal inoculation contained live *E. coli*, by 10–15 % in tubes pre-incubated with bacteria, then heated and inoculated in the absence of viable *E. coli* and by 2–6 % in tubes incubated (whether pre-incubated in the absence of bacteria or not) without added *E. coli*. The addition of the redox indicator resazurin (0·0001 %) showed that although the indicator was reduced in all tubes, in the tubes with living bacteria it reduced more rapidly and re-oxidized more slowly on exposure to air than in those which contained heated or no *E. coli*. These results suggest that a low redox potential was important for protozoal growth in the given conditions.

Effect of addition of Escherichia coli after 48 hr. Protozoal cultures incubated without added bacteria contained about 50×10^7 viable bacteria/ml. after 48 hr. It was therefore of interest to measure the rate of disappearance of a bacterium which did not grow under these conditions, namely *Escherichia coli*, in order to obtain an estimate of the rate at which other bacteria were being engulfed. In one experiment 49×10^7 *E. coli* organisms/ml. were added at 48 hr to a culture that contained $1·87 \times 10^4$ protozoa and 60×10^7 bacteria/ml. After 6 hr and 25 hr the numbers of viable

E. coli organisms were 19×10^7 and $< 10^6$/ml., respectively and the number of other bacteria were 50×10^7/ml. after 25 hr. The number of viable *E. coli* organisms in control tubes incubated without protozoa for 25 hr decreased by less than 15%.

DISCUSSION

The results reported show that the engulfment and digestion of bacteria by *Entodinium caudatum* is a more important process than was previously thought and that the protozoa engulf most bacteria with which they come in contact. Theoretically, when the protozoa are offered *Escherichia coli* and, for example, a Bacterium X which was ten times the size of *E. coli*, Bacterium X would take up the space of ten *E. coli* organisms in the gastric sac and the presence of one Bacterium X to ten *E. coli* organisms in the medium would decrease the uptake of *E. coli* to 50% if neither bacterium were taken up preferentially. Since the efficiency of a bacterium in decreasing the total uptake of *E. coli* was found experimentally to be proportional to its size, this suggests that *Entodinium caudatum* engulfed different bacteria in the proportion in which they were present in the medium. It is of interest that *Streptococcus bovis*, Bacterium D and Bacterium 31, which were isolated from the sheep rumen or from protozoal cultures, were not engulfed preferentially to organisms isolated from other habitats.

Although several species of bacteria stimulated the growth of protozoa more than the bacteria which grew from those associated with the protozoal inoculum, it is not claimed that under these conditions, *Escherichia coli*, for example, was the only bacterium taken up. It seems certain that the other bacteria growing in the medium were also engulfed and it is hoped that the results obtained with *E. coli* are representative of what occurred with other bacteria. The rapid disappearance of *E. coli* added to growing cultures after incubation for 48 hr suggests that other bacteria were also being rapidly engulfed and that the number present represents a balance between multiplication of the bacteria and their engulfment by the protozoa. Although this stimulatory effect occurred only with living organisms it was obtained with micro-organisms of widely differing properties, namely a short Gram-negative aerobic nutritionally non-exacting rod (*E. coli*), a large Gram-positive anaerobic nutritionally-exacting rod (*Clostridium welchii*) and a yeast. This suggested that one or more of the following three factors may be important. Since the protozoa require a low redox potential in the medium for survival and growth, all the micro-organisms when alive would probably reduce the potential below that obtainable by the use of cysteine. Secondly, if the protozoa require solid nucleic acid and protein particles in order to obtain a sufficiently high concentration of purines, pyrimidines and amino acids in the gastric sac for nucleic acid and protein synthesis, then living or dead bacteria could supply this requirement. This may be of particular importance in the rumen where the concentration of free amino acids is low but the concentration of bacteria high. Thirdly, either some low molecular weight compounds normally found in the metabolic 'pool' of bacteria or a bacterial enzyme may be required and both these would be lost on boiling. However, a requirement for any proteinaceous material is thought to be unlikely in view of the rapid digestion of protein which occurs in the gastric sac.

I thank Miss E. V. Holgate for her valuable technical assistance.

REFERENCES

BAILEY, R. W. & OXFORD, A. E. (1958). A quantitative study of the production of dextran from sucrose by rumen strains of *Streptococcus bovis*. *J. gen. Microbiol.* **19**, 130.

BRYANT, M. P. & ROBINSON, I. M. (1961). An improved non-selective culture medium for ruminal bacteria and its use in determining diurnal variation in numbers of bacteria in the rumen. *J. Dairy Sci.* **44**, 1446.

COLEMAN, G. S. (1958). Maintenance of oligotrich protozoa from the sheep rumen *in vitro*. *Nature, Lond.* **182**, 1104.

COLEMAN, G. S. (1960*a*). The cultivation of sheep rumen oligotrich protozoa *in vitro*. *J. gen. Microbiol.* **22**, 555.

COLEMAN, G. S. (1960*b*). Effect of penicillin on the maintenance of rumen oligotrich protozoa. *Nature, Lond.* **187**, 518.

COLEMAN, G. S. (1962). The preparation and survival of almost bacteria-free suspensions of *Entodinium caudatum*. *J. gen. Microbiol.* **28**, 271.

GUTIERREZ, J. & DAVIS, R. D. (1959). Bacterial ingestion by the rumen ciliates *Entodinium* and *Diplodinium*. *J. protozool.* **6**, 222.

POTTER, V. R. & ELVEHJEM, C. A. (1936). A modified method for the study of tissue oxidations. *J. biol. Chem.* **114**, 495.

ROBERTS, R. B., ABELSON, P. H., COWIE, D. B., BOLTON, E. G. & BRITTEN, R. J. (1955). Studies on Biosynthesis in *Escherichia coli*. *Publ. Carneg. Instn*, no. 607.

SCHNEIDER, W. C. (1945). Phosphorus compounds in animal tissues. 1. Extraction and estimation of desoxypentose nucleic acid and of pentose nucleic acid. *J. biol. Chem.* **161**, 293.

J. gen. Microbiol. (1964), 37, 225–229
Printed in Great Britain

A Role for a Sterol and a Sterol Precursor in the Bacterium *Rhodopseudomonas palustris*

By S. AARONSON
Haskins Laboratories, New York, N.Y. 10017, U.S.A.

(*Received* 5 *June* 1964)

SUMMARY

The multiplication of the purple photosynthetic bacterium *Rhodopseudomonas palustris* is inhibited by the hypocholesteraemic agents benzmalecene and triparanol. This inhibition is annulled by oleic acid, ergosterol, squalene, farnesol and lanosterol, in that order, but not by mevalonic acid, geranyl acetate, palmitic or stearic acids. Only oleic acid annuls the inhibition in higher concentrations of the inhibitors. Ergosterol and squalene, while not effective against higher concentrations of inhibitor, spared the annulment by oleic acid. An unknown interaction between oleic acid and sterols or their precursors is suggested.

INTRODUCTION

The existence of sterols in bacteria is questionable. The evidence was reviewed by Asselineau (1962) and Fiertel & Klein (1959); relatively few bacteria have been analysed. Various compounds block different sites in the synthesis of cholesterol in mammals (Holmes & Di Tullio, 1962). Two of these, benzmalecene and triparanol, inhibited protozoa (Aaronson, Bensky, Shifrine & Baker, 1962) and have been studied here to see whether they uncover sterol or sterol-related functions in bacteria, much as sulphanilamide uncovered *p*-aminobenzoic acid (Woods, 1940).

METHODS

Rhodopseudomonas palustris was grown by methods described elsewhere (Keane, Zahalsky, Hutner & Lubart, 1963). Experimental cultures were incubated for 5 days at 28° under incandescent and fluorescent lamps.

The chemicals used were commercial grades; the fatty acids (99 % pure by gas–liquid chromatography) were purchased from Applied Science Laboratories, State College, Pa., U.S.A., or the Hormel Institute, Austin, Minn., U.S.A.

Benzmalecene was supplied by Dr D. Hendlin (Merck, Sharp & Dohme Research Laboratories, Rahway, N. J.) and triparanol by Dr F. J. Murray (W. S. Merrell Co., Cincinnati, Ohio, U.S.A.). Benzmalecene was dissolved in alkaline water and triparanol was dissolved in 95 % (v/v) ethanol in water. The concentrations of ethanol used were not toxic.

RESULTS

Inhibition of *Rhodopseudomonas palustris* multiplication by benzmalecene and triparanol was annulled by crude lipid preparations: crude lecithin and TEM-4 (the diacetyl tartaric acid ester of tallow glycerides) and by the following compounds:

oleic acid, ergosterol, squalene, farnesol, lanosterol in that order, but not by mevalonic acid, geranyl acetate, palmitic or stearic acids (see Table 1). Oleic acid was the most effective compound in annulling inhibitions at the higher concentrations of the inhibitors, while squalene and ergosterol were almost as effective as oleic acid at low inhibitory concentrations but not at higher concentrations of benzmalecene and triparanol (Tables 2, 3). Only oleic acid was effective at higher concentrations. At a higher concentration of benzmalecene, squalene or ergosterol while ineffective by themselves spared the annulment by oleic acid. Similar results were obtained with triparanol.

Table 1. *Annulment by lipids of inhibition of growth of Rhodopseudomonas palustris produced by hypocholesteraemic agents*

		Conc. (mM)					
		Benzmalecene			Triparanol		
		0·08	0·16	0·26	0·07	0·14	0·23
		Optical extinction reading					
Additions	Conc.						
None	—	1·43	0·22	0	1·64	0	0
TEM	1·0 mg. %	1·76	0·84	0·13	1·95	1·48	0
	3·0	1·83	0·94	0·21	1·96	1·55	0
	20·0	1·92	1·54	0·98	1·98	1·98	1·56
	30·0	1·96	1·85	1·58	1·98	1·98	1·85
Lecithin	1·0 mg.%	1·76	0·31	0	1·95	0·83	0
	3·0	1·84	0·72	0	1·97	1·34	0
	10·0	1·92	1·72	1·64	1·99	1·88	0·04
	20·0	1·98	1·96	1·85	1·99	1·95	0·99
	30·0	2·00	1·98	1·89	1·99	1·97	1·66
Oleic acid	0·04 mM	1·89	0·26	0	1·86	0·53	0·06
	0·11	1·72	0·59	0·18	1·90	1·36	0·33
	0·35	1·86	1·39	0·90	1·86	1·65	1·59
	0·70	1·94	1·70	1·45	1·79	1·65	1·61
	1·05	1·78	1·63	1·61	1·87	1·75	1·79
Squalene	0·04	1·70	0·21	0	1·80	0·49	0·07
	0·35	1·94	1·53	0·11	1·77	0·67	0·05
Farnesol	0·04	1·16	0	0	1·92	0	0·04
	0·35	1·78	0·29	0	1·74	0·72	0·13
Lanosterol	0·04	1·39	0	0	1·27	0	0
	0·35	1·51	0	0	1·62	0·52	0·06
Ergosterol*	0·04	1·46	0·14	0	1·88	0·25	0
	0·35	1·92	1·37	0·22	1·95	1·75	0·15

* Crude.

DISCUSSION

Evidence for sterols in bacteria is meagre and unsatisfactory; this information derives from rather non-specific reactions, e.g. the Liebermann–Burchard (L-B) reaction, and on bacteria grown in ill-defined and often sterol-containing media. Bacteria which do not require sterols for growth may nevertheless accumulate unchanged sterols (Smith & Rothblatt, 1960; Rebel & Mandel, 1962). Moreover, the methods used to detect sterols in bacteria were not flawless: saturated sterols do not give a colour reaction (L-B) and slight structural changes in the sterol

molecule render it unprecipitable by digitonin (Bergmann, 1962). The availability of compounds which inhibit sterol biosynthesis in mammals (Holmes & Di Tullio, 1962) allowed us to test the sterol-inhibiting properties of these compounds on bacteria in the hope of demonstrating a sterol requirement by inhibition analysis. In mammals benzmalecene inhibits early in sterol synthesis at the reaction sequence which converts isopentylpyrophosphate into squalene; triparanol inhibits a terminal step: reduction of desmosterol to cholesterol (Holmes & Di Tullio, 1962).

Table 2. *Interaction between oleic acid and squalene in annulling inhibition of growth of Rhodopseudomonas palustris*

		Squalene (mM)			
		0	0·035	0·105	0·35
Compound	Conc. (mM)	Optical extinction reading			
Benzmalecene	0·16	0·47	0·65	1·53	2·13
Benzmalecene + oleic acid	0·04	0·80	1·68	2·24	2·46
	0·11	1·46	1·88	2·38	2·54
	0·35	1·86	2·23	2·58	2·62
	1·05	2·13	2·40	2·50	2·50
Benzmalecene	0·26	0	0	0	0
Benzmalecene + oleic acid	0·04	0·18	0·42	0·76	2·39
	0·11	0·28	0·70	0·64	2·57
	0·35	1·17	1·29	1·75	2·56
	1·05	1·60	1·82	1·97	2·40
Triparanol	0·14	0	0·16	0·47	0·97
Triparanol + oleic acid	0·04	0·96	1·59	1·82	1·94
	0·11	2·50	2·46	2·51	2·46
	0·35	2·64	2·58	2·61	2·55
	1·05	2·49	2·53	2·40	2·32
Triparanol	0·23	0·08	0·06	0·06	0·04
Triparanol + oleic acid	0·04	0·02	0·05	0·08	0·06
	0·11	0·30	0·55	0·46	0·55
	0·35	2·58	2·57	2·58	2·46
	1·05	2·48	2·48	2·44	2·38

In *Rhodopseudomonas palustris* the inhibition produced by low concentrations of benzmalecene or triparanol was annulled almost as well by squalene and ergosterol as by oleic acid. At higher concentrations of these inhibitors only oleic was effective; squalene and ergosterol, while inactive alone, spared the oleic acid. These results suggest a metabolic role for the ergosterol and the sterol precursor, squalene, in this bacterium as well as a curious and as yet unexplained interaction between oleic acid and the sterols. Jensen (1962) identified a triterpene, paluol, in two strains of *R. palustris*. Possibly benzmalecene and triparanol inhibit the synthesis of paluol, an inhibition presumably annulled by squalene or ergosterol. Isotopically-labelled lipids need to be used before one can assign an indispensable function for sterols and explain the sterol–oleic acid interaction in *R. palustris*.

There is as yet no explanation for the role of oleic acid in the synthesis of non-saponifiable lipids. Kodicek (1959) observed that ^{14}C-fatty acids from yeast were incorporated more effectively (11·9 %) into the non-saponifiable lipids of yeast than

was ^{14}C-acetate (1·7 %), despite the apparent inability of the yeast to degrade the fatty acids to acetate. Bloch & Amdur (Asselineau, personal communication, 1962) observed similarly that ^{14}C-acetate was not taken up at all in the non-saponifiable fraction of *Azotobacter chroococcum* or *Escherichia coli*.

Table 3. *Interaction between oleic acid and ergosterol in annulling inhibition of growth of Rhodopseudomonas palustris*

		Ergosterol* (mM)			
		0	0·035	0·105	0·35
Compound	Conc. (mM)	Optical extinction reading			
Benzmalecene	0·16	0·15	0·26	0·34	0·51
Benzmalecene + oleic acid	0·04	0·27	0·48	0·74	1·52
	0·11	0·81	1·71	2·57	2·62
	0·35	1·43	2·34	2·42	2·57
	1·05	1·77	2·44	2·44	2·45
Benzmalecene	0·26	0·03	0·08	0·16	0·39
Benzmalecene + oleic acid	0·04	0·10	0·18	0·40	0·67
	0·11	0·40	0·62	1·31	1·74
	0·35	0·91	1·57	2·24	2·54
	1·05	1·43	1·76	2·24	2·16
Triparanol	0·14	0·10	1·74	1·86	1·66
Triparanol + oleic acid	0·04	0·97	2·21	2·31	2·18
	0·11	2·42	2·45	2·49	2·26
	0·35	2·60	2·58	2·63	2·49
	1·05	2·30	2·25	2·37	2·24
Triparanol	0·23	0·06	0·06	0·08	0·18
Triparanol + oleic acid	0·04	0·09	0·08	0·06	0·20
	0·11	0·12	0·27	0·45	0·45
	0·35	2·58	2·54	2·68	2·73
	1·05	2·65	2·52	2·42	2·56

* Pure (homogeneous by thin-layer chromatography).

Holz, Erwin, Wagner & Rosenbaum (1962) found that mixtures of long-chain fatty acids (oleic, palmitic, stearic) and a synthetic phospholipid spared sterols in the sterol-requiring ciliate *Tetrahymena setifera* HZ-1. *T. pyriformis* synthesizes a triterpene but no sterol (Mallory, Gordon & Conner, 1963) and is inhibited by triparanol. This inhibition is annulled by sterols or long-chain fatty acids (oleic, palmitic, stearic; Holz, Erwin, Rosenbaum & Aaronson, 1962). Further work may elucidate the peculiar relationship between fatty acids, sterols and terpenes.

This work was supported by grant GM 09103 from the National Institutes of Health, U.S. Public Health Service.

REFERENCES

AARONSON, S., BENSKY, B., SHIFRINE, M. & BAKER, H. (1962). Effect of hypocholesteremic agents on protozoa. *Proc. Soc. exp. Biol., N.Y.* **109**, 130.

ASSELINEAU, J. (1962). *Les Lipides Bactériens*. Paris: Hermann et Cie.

BERGMANN, W. (1962). Sterols: their structure and distribution. *Comparative Biochemistry*, III A, pp. 103–162. New York: Academic Press.

FIERTEL, A. & KLEIN, H. P. (1959). On sterols in bacteria. *J. Bact.* **78**, 738.

HOLMES, W. L. & DI TULLIO, N. W. (1962). Inhibitors of cholesterol biosynthesis which act at or beyond the mevalonic acid stage. *Amer. J. clin. Nutr.* **10**, 310.

HOLZ, G. G., JUN., ERWIN, J., ROSENBAUM, N. & AARONSON, S. (1962). Triparanol inhibition of Tetrahymena, and its prevention by lipids. *Arch. Biochem. Biophys.* **98**, 312.

HOLZ, G. G., JUN., ERWIN, J., WAGNER, B. & ROSENBAUM, N. (1962). The nutrition of *Tetrahymena setifera* HZ-1; sterol and alcohol requirements. *J. Protozool.* **9**, 359.

JENSEN, S. L. (1962). The constitution of some bacterial carotenoids and their bearing on biosynthetic problems. *Kgl. Norske Videnskabeis Selskabs Skrifter*, nr. 8, 1–199.

KEANE, M., ZAHALSKY, A. C., HUTNER, S. H. & LUBART, K. J. (1963). Defined growth media for *Rhodopseudomonas palustris* and *R. spheroides*. *Studies on Microalgae and Photosynthetic Bacteria*, pp. 163–169. Tokyo: University of Tokyo Press.

KODICEK, E. (1959). Biosynthesis of yeast sterols and the preparation of C^{14}-labelled vitamin D_2. In *Biosynthesis of Terpenes and Sterols*. Ed. by G. E. W. Wolstenholme & C. M. O'Connor, pp. 173–183. Boston: Little Brown and Co.

MALLORY, F. B., GORDON, J. T. & CONNER, R. L. (1963). The isolation of a pentacyclic triterpenoid alcohol from a protozoan. *J. Amer. chem. Soc.* **85**, 1362.

REBEL, G. & MANDEL, P. (1962). Sur la présence de cholestérol dans les formes L dérivées du bacille *Proteus*. *C.r. hebd. Séanc. Acad. Sci., Paris*, **255**, 2684.

SMITH, P. F. & ROTHBLATT, G. H. (1960). Incorporation of cholesterol by pleuropneumonia-like organisms. *J. Bact.* **80**, 842.

WOODS, D. D. (1940). The relation of *p*-aminobenzoic acid to the mechanism of the action of sulphanilamide. *Brit. J. exp. Path.* **21**, 74.

J. gen. Microbiol. (1964), 37, 231–249
Printed in Great Britain

A Note on Koch & Schaechter's Hypothesis about Growth and Fission of Bacteria

BY E. O. POWELL

Microbiological Research Establishment, Porton Down, Salisbury, Wiltshire

(*Received* 6 *June* 1964)

SUMMARY

The hypothesis of Koch & Schaechter (1962) in its general form gives rise to mathematical relationships of great complexity. The formulae are simplified when certain conditions are met; the most important condition is that the smallest organism about to divide should not be smaller than the largest newly formed organism. Real cultures often satisfy the condition, and they can then fairly be used to test the hypothesis.

INTRODUCTION

Previous attempts to account for the variability of the generation times of individual bacteria have been based on the supposition that the generation time is directly determined by a nexus of primitive events, essentially chemical in nature and involving few molecules (Rahn, 1932; Kendall, 1948). In two classical papers (Adolph & Bayne-Jones, 1932; Bayne-Jones & Adolph, 1932) Adolph and Bayne-Jones suggested that generation time was primarily determined by size at the time of fission; this suggestion seems to have been prompted only by the fact that, in the early stages of a batch culture, the mass growth rate begins to increase before the number growth rate, and, in the late stages, the mass growth rate decreases before the number growth rate. Koch & Schaechter (1962) put forward a clearly formulated hypothesis of the same kind; they suppose that the primitive events, whatever their nature, determine primarily the size of the organism at its termination; the size (i.e. mass or volume) is a stochastic variable whose target is a certain critical value characteristic of a given species in a given environment. With the addition of a few natural and moderate assumptions, the pattern of generation times follows.

In their exposition Koch & Schaechter make a number of approximations which they do not justify explicitly and quantitatively. The purpose of this present note is to give a more detailed account of the consequences of the hypothesis. It will appear that the hypothesis implies a pattern of growth involving mathematical relationships of great complexity, but that radical simplification is possible under certain conditions which are often met, with a measurable degree of error, by real cultures.

Koch & Schaechter postulate, first, that the mass growth rate of the individual organism is a constant, say ν, the same for all organisms in the steady state (I use ν instead of Koch & Schaechter's λ since the constant must, in the steady state, be equal to both the mass and number growth rate of the whole culture). The same will

be true of the volume growth rate if the density remains constant, and less exactly of the length of rod-like organisms if both the density and diameter remain constant. In practice it is impossible to measure the mass or diameter with any accuracy, and I shall use x to denote 'size' in the sense of mass, volume or length, leaving the appropriate interpretation for separate discussion or investigation. Then for any individual

$$\frac{dx}{dt} = \nu x; \quad x \propto e^{\nu t}.$$

Secondly, they postulate that the sizes of organisms at termination (x_t) are scattered about a critical value, and that the x_t are independent of one another. They assume further that the distribution of x_t is normal, but this is an unnecessary and indeed unrealistic restriction. Thirdly, they postulate that when an organism divides the two daughter organisms are nearly equal in size. In some contexts, it is a reasonable approximation to assume that the daughters are exactly equal; in others the differences must be taken into account.

In the sequel I have varied Koch & Schaechter's notation in order to avoid confusion with my own usage.

I make use of two principles which are not widely known:

(*a*) A convenient way is needed of determining the expectation ($\mu'_r(\log v)$) of the rth power of the logarithm of a variate v which takes only positive values. If v has a frequency function $f(v)$ the Mellin transform

$$\mathfrak{M}(s;f) = \int_0^\infty v^{s-1} f(v)\, dv$$

is a generating function for $\mu'_r(\log v)$ in that

$$\mu'_r(\log v) = \frac{d^r}{ds^r}\{\mathfrak{M}(s;f)\}|_{s=1}.$$

Similarly, $\log\{\mathfrak{M}(s;f)\}$ is a generating function for the cumulants $\kappa_r(\log v)$. These statements are a simple transformation of, and are guaranteed by, equivalent statements which could be made in terms of the more conventional characteristic functions (Fourier complex transforms). The form given is direct in application since tables of Mellin transforms exist (Erdélyi, 1954). The Mellin transform differs from the ordinary moment-generating function of $\log v$ only in having the index $s-1$ instead of s.

(*b*) If a variate v takes only positive values, and its coefficient of variation is $c(v)$, then

$$\text{var} \log v \simeq c^2(v)$$

provided (i) that $c(v)$ is small, (ii) that the higher moment ratios $\mu_r/(\mu'_1)^r$ of v are all small compared with $c(v)$.

Inferences from Koch & Schaechter's hypothesis

The preliminary difficulty

Before proceeding, we must resolve a difficulty not discussed by Koch & Schaechter. It appears as soon as we attempt to derive the generation-time distribution in a naïve way.

Let $l(x_t)$ be the frequency function of the size of organisms at termination and put

$$L_-(x_t) = \int_{x_t}^{\infty} l(\xi)\, d\xi.$$

Assume at first that sisters are exactly equal in size at inception (x_i). Then the frequency function $l_i(x_i)$ of x_i is $2l(2x_i)$. Write $f_s(\tau)$ for the frequency function of generation times τ and $F_{s-}(\tau)$ for

$$\int_{\tau}^{\infty} f_s(\xi)\, d\xi.$$

Organisms whose generation times are greater than τ are those which, whatever their initial size, are of a size greater than $x_i\, e^{\nu\tau}$ at termination, since for each organism $x_t = x_i\, e^{\nu\tau}$. Thus

$$F_{s-}(\tau) = \int_0^{\infty} 2l(2x_i)\, L_-(x_i\, e^{\nu\tau})\, dx_i. \tag{1}$$

By differentiation,

$$f_s(\tau) = 2\nu\, e^{\nu\tau} \int_0^{\infty} x_i\, l(2x_i)\, l(x_i\, e^{\nu\tau})\, dx_i; \tag{2}$$

in this expression it is convenient to replace x_i by $\frac{1}{2}x_t$:

$$f_s(\tau) = \tfrac{1}{2}\nu\, e^{\nu\tau} \int_0^{\infty} x_t\, l(x_t)\, l(\tfrac{1}{2}x_t\, e^{\nu\tau})\, dx_t. \tag{3}$$

The substitution $\xi = \sqrt{2}x_i\, e^{\frac{1}{2}\nu\tau}$, on the other hand, yields

$$f_s(\tau) = \nu \int_0^{\infty} \xi l\left[\xi \exp\left\{-\frac{\nu}{2}\left(\tau - \frac{\log 2}{\nu}\right)\right\}\right] l\left[\xi \exp\left\{\frac{\nu}{2}\left(\tau - \frac{\log 2}{\nu}\right)\right\}\right] d\xi, \tag{4}$$

which shows that $f_s(\tau)$ is symmetrical about $\tau = (\log 2)/\nu$, whatever the form or degree of dispersion of x_t. It follows that the arithmetic mean of τ is $(\log 2)/\nu$.

Now if we allow that the possible range of $l(x_t)$ is not limited, we encounter negative generation times in those instances where x_t is less than the corresponding x_i, of which it is postulated to be independent; in fact the integral of (3) over positive τ only is less than unity. Powell's (1955, 1958) discussion shows that there is nothing irrational in this so far as cell-wall fission (*o*-fission) is concerned, and that negative generation times can very occasionally be recognized; they are awkward to deal with, however, since a generation time wrongly recorded as positive implies that another interval will be recorded which is in fact the sum of two generation times; and higher degrees of complication can conceivably occur.

We can deal with the situation in an obvious way by assuming that instances of negative generation time are negligibly few, but the assumption is uncomfortably vague until we have some means of assessing the error involved in making it. In fact, Schaechter, Williamson, Hood & Koch (1962) noted that of the organisms they examined less than 1 % contained four nuclei at any time. The progeny of such cells only could (but would not necessarily) have negative generation times. We are on firmer ground, however, if we try to find what observed pattern of growth would result from Koch & Schaechter's postulates, the intervals between successive fissions being called (positive) generation times and labelled τ; that is, if we accept the observations at their face value and disregard questions of internal structure.

According to the hypothesis, an organism has an intrinsic tendency to fission

which at a given time depends only on its size at that time and not, in particular, on its size at inception. With this intrinsic tendency we can associate a hypothetical frequency function $\lambda(x_t)$ such that, if an organism has reached the size x_t, its chance of dividing before it reaches a size x_t+dx_t is

$$\lambda(x_t)\,dx_t\Big/\int_{x_t}^{\infty}\lambda(\xi)\,d\xi.$$

Now if size at termination is biologically independent of size at inception, it will not in general be statistically independent of it. Just as a sexagenarian is more likely to reach the age of 70 than is a young man (other things being equal), so an organism whose size is already x is more likely to attain a given size x_t ($x_t > x$) at termination than one whose size is less than x. The realized and observable distribution of x_t will not be $\lambda(x_t)$, but, *for a given* x_i, will be

$$\lambda(x_t)/\Lambda_-(x_i), \tag{5}$$

where

$$\Lambda_-(x_i) = \int_{x_i}^{\infty}\lambda(\xi)\,d\xi.$$

If an organism is of size x_i at inception, the probability of its size at termination being greater than x_t is

$$\Lambda_-(x_t)/\Lambda_-(x_i) \quad (x_t > x_i),$$

or

$$1 \qquad (x_t < x_i).$$

But the probability of realizing x_i depends in the same way on the size at inception of the mother of the organism in question. We therefore introduce the realized distributions $l(x_t)$ of size at termination and $l_i(x_i)$ of size at inception. Then the observed frequency of fission at a size greater than x_t will be

$$L_-(x_t) = \int_{x_t}^{\infty} l(\xi)\,d\xi = \int_0^{x_t}\frac{\Lambda_-(x_t)}{\Lambda_-(x_i)}\,l_i(x_i)\,dx_i + \int_{x_t}^{\infty} l_i(x_i)\,dx_i, \tag{6}$$

the last term corresponding to the frequency with which x_i is greater than a given x_t (when fission necessarily occurs at a size greater than x_t). By differentiating twice we find in turn

$$\frac{l(x_t)}{\lambda(x_t)} = \int_0^{x_t}\frac{l_i(x_i)}{\Lambda_-(x_i)}\,dx_i; \tag{7}$$

$$\frac{d}{dx_t}\left\{\frac{l(x_t)}{\lambda(x_t)}\right\} = \frac{l_i(x_t)}{\Lambda_-(x_t)}. \tag{8}$$

We can now express λ in terms of l by treating (8) as a second-order differential equation in $\Lambda_-(x_t)$. Putting temporarily

$$W = \frac{1}{l(x_t)}\frac{dl(x_t)}{dx_t}, \quad V = \frac{l_i(x_t)}{l(x_t)}, \quad Z = \frac{\lambda(x_t)}{\Lambda_-(x_t)},$$

(8) becomes

$$\frac{dZ}{dx_t} + Z^2(V-1) - ZW = 0,$$

a Bernoulli equation soluble by conventional methods. A first integral is

$$\frac{\lambda(x_t)}{\Lambda_-(x_t)} = l(x_t)\Big/\left\{\int_{x_t}^{\infty} l(\xi)\,d\xi - \int_{x}^{\infty} l_i(\xi)\,d\xi + A\right\}, \tag{9}$$

where A is a constant. Now $\Lambda_-(0) = 1$, and so the integral in (7) converges at the lower limit because $l_i(x_i)$ is a frequency function. Hence

$$\lim_{x_t \to 0} \frac{l(x_t)}{\lambda(x_t)} = 0.$$

Assuming for a moment that $\lambda(0) = 0$, rearrangement of (9) shows that A will then be zero, and

$$\frac{\lambda(x_t)}{\Lambda_-(x_t)} = l(x_t) \bigg/ \left\{L_-(x_t) - \int_{x_t}^{\infty} l_i(\xi)\, d\xi\right\}. \tag{10}$$

The formal simplicity of this expression belies its real complexity, and an attempt to obtain an explicit formula for Λ_- or λ by a further integration immediately raises difficulties of convergence. We can avoid these difficulties if we suppose that there is a non-zero lower limit to the size of an organism. This supposition is entirely acceptable biologically, and, since it also implies $\lambda(0) = 0$, we can take (10) as a mathematically valid relation. It turns out that (10) as it stands is more useful than an explicit expression for $\lambda(x_t)$.

In particular, when organisms can be assumed to divide into daughters of equal size, we have

$$l_i(x_i) = 2l(2x_i)$$

and (10) becomes

$$\frac{\lambda(x_t)}{\Lambda_-(x_t)} = \frac{l(x_t)}{L_-(x_t) - L_-(2x_t)}. \tag{11}$$

Fission into daughters of unequal size

The obvious way to describe the relation between size at inception and size at termination is to introduce a bivariate distribution, $\mathsf{L}(x_t, x_i)$, say, in which it is to be understood that x_i is the size at inception of a daughter, x_t the size at termination of its mother. Then the frequency function of x_i irrespective of x_t is

$$\int_{x_i}^{\infty} \mathsf{L}(x_t, x_i)\, dx_t$$

(since x_t cannot be less than x_i), and

$$l(x_t) = \int_0^{x_t} \mathsf{L}(x_t, x_i)\, dx_i.$$

Thus L is very unsymmetrical; it can occupy only the area between the lines $x_i = 0$, $x_i = x_t$ in the x_i, x_t plane (Fig. 1*a*). Since every fission gives rise to two daughters and since, if the size of either is called x_i, the size of the other is $x_t - x_i$, the distribution of x_i for any given x_t is symmetrical with mean $\frac{1}{2}x_t$. The regression of x_i on x_t is $x_i = \frac{1}{2}x_t$. Suppose now we introduce a new variable p:

$$p = \frac{x_i\,(\text{daughter})}{x_t\,(\text{mother})}.$$

Then again the distribution of p is symmetrical; p and $1-p$ are equally frequent, and the possible range of p is from 0 to 1 for any x_t. The joint probability element $\mathsf{L}(x_t, x_i)\, dx_t\, dx_i$ becomes $\mathsf{L}(x_t, px_t)x_t\, dx_t\, dp$. On account of the symmetry of the p-distribution $\bar{p} = \frac{1}{2}$ and the ordinary product-moment correlation coefficient

$\rho(x_t, p)$ is zero. But it does not follow that p is independent of x_t in any more fundamental sense. In multicellular species for example, extreme dissymmetry of fission may be relatively commoner in very long organisms where the nearly simultaneous development of several cross-walls provides opportunity for fission at a number of discrete points (Powell, 1955). Then the joint distribution of p and x_t might take some such form as Fig. 1*b*. On the other hand, in the unicellular species investigated by Schaechter *et al.* (1962) and Powell & Errington (1963*b*) the dispersion of p was very small. Accordingly Koch & Schaechter (1962) assumed that the distribution of p could be represented by a single frequency function, say $k(p)$, the same for all x_t.

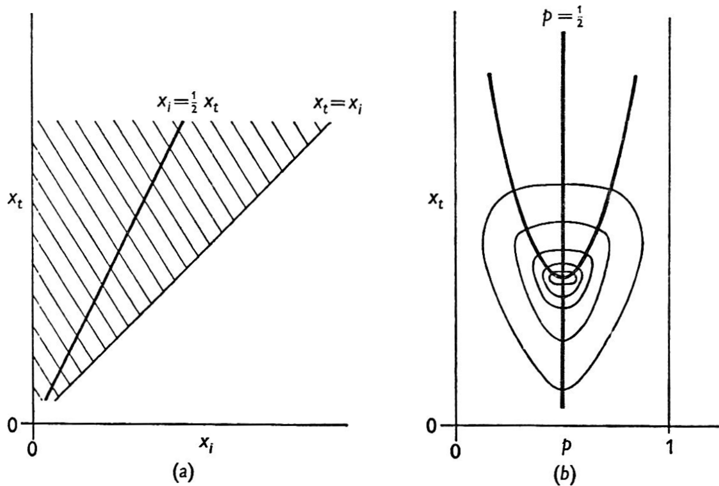


Fig. 1*a*. The joint distribution of x_t (length at termination of mother) and x_i (length at inception of daughter) occupies the shaded sector. The regression of x_i on x_t is $x_i = \frac{1}{2}x_t$. Fig. 1*b*. The joint distribution of p $(= x_i/x_t)$ and x_t occupies the area between $p = 0$ and $p = 1$. The regression of p on x_t is $p = \frac{1}{2}$. The heavy parabolic line is a possible form for the regression of x_t on p which still leaves the correlation zero. The contours of the correlation surface are indicated schematically.

With this assumption, we can easily find a general expression for the distribution $l_i(x_i)$ of size at inception. The probability that an organism should be of a size greater than x_i at inception is the probability that, whatever the size x_t of its mother at termination, the associated value of p should be greater than x_i/x_t, i.e.

$$\int_{x_i}^{\infty} l_i(\xi)\,d\xi = \int_{x_i}^{\infty}\int_{x_i/x_t}^{1} l(x_t)\,k(p)\,dp\,dx_t$$

$$= \int_0^1\int_{x_i/p}^{\infty} l(x_t)k(p)\,dx_t\,dp$$

$$= \int_0^1 L_-\left(\frac{x_i}{p}\right)k(p)\,dp,$$

whence

$$l_i(x_i) = \int_0^1 l\left(\frac{x_i}{p}\right)\frac{k(p)}{p}\,dp, \tag{12}$$

and (10) becomes

$$\frac{\lambda(x_t)}{\Lambda_(x_t)} = l(x_t) \bigg/ \left\{L_(x_t) - \int_0^1 L_\left(\frac{x_t}{p}\right) k(p)\, dp\right\}; \tag{13}$$

this agrees with (11) since, when p is not dispersed, $1/p$ takes only the value 2.

No sufficient data yet exist to show that $k(p)$ and its parameters are or are not independent of x_t, but if (12) is adopted it should be remembered that an additional assumption has been made, though perhaps not a quantitatively important one.

The steady-state distribution of size

The family tree generated by reproduction from a single organism is ideally one in which all generation times are alike equal to $(\log 2)/\nu$. Actual times of fission, because of the dispersion of size at termination, do not occur simultaneously in every generation, but, under the assumption of fission into equal daughters, are

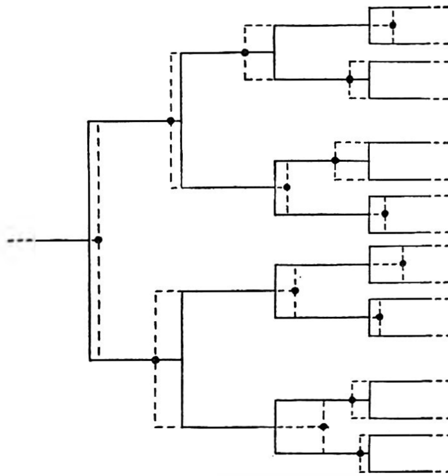


Fig. 2. The continuous line is an idealized family tree in which all generation times (horizontal segments) are alike, and all fissions in any one generation occur simultaneously. Under Koch & Schaechter's hypothesis, actual times of fission (heavy dots) are dispersed about the ideal with a variance which is the same in every generation.

scattered about their mean with a variance ν^{-2} var $\log x_t$ which is the same for every generation (Fig. 2). A culture started from one organism or from a collection of organisms all of the same size would thus maintain a fixed degree of synchrony for an indefinitely long period; the number growth rate $d \log \mathrm{n}/dt$ would be a strictly periodic function.

Among students of synchrony, the most optimistic draughtsmen know that the condition does not long persist. Qualitatively, it is easy to explain the lack of persistence. In unicellular organisms, fission usually results in sisters of nearly equal size, but there are occasional disparities which though not extravagant are detectable by simple inspection. If in a large tree such as that of Fig. 2 we imagine that a few fissions result in sisters of unequal size, the subtrees stemming from those sisters will be similar to the tree of the ancestral organism but out of phase with it. Repetition of the process will, we can suppose, lead to a culture in which all

synchrony has been lost and the population of organisms has an unchanging size and age distribution. We have then to inquire what the steady state must be.

In view of the rarity with which pairs of markedly unequal sisters are formed, it is perhaps permissible, once the steady state has been reached, to assume that fission is symmetrical. With this restriction, Koch & Schaechter derive a differential-difference equation for the frequency function $\mathscr{L}(x_0)$ of the size of the organisms in the steady state. They also assume that, if an organism has attained a size x, the probability that it will divide when its size is in the range $x_t, x_t + dx_t$ is $\lambda(x_t)\, dx_t/\Lambda_-(x)$. That is, they make use implicitly of the assumption (5) on which we have based (11). Their equation is

$$\frac{d}{dx_0}\left\{x_0^2\mathscr{L}(x_0)\right\} = 2(2x_0)^2\mathscr{L}(2x_0)\frac{\lambda(2x_0)}{\Lambda_-(2x_0)} - x_0^2\mathscr{L}(x_0)\frac{\lambda(x_0)}{\Lambda_-(x_0)}. \tag{14}$$

Koch & Schaechter's reasoning so far seems to be impeccable, but they go on to suggest that

$$\mathscr{L}(x_0) = \frac{C}{x_0^2}\int_{x_0}^{2x_0}\lambda(\xi)\, d\xi \quad (C \text{ a constant}) \tag{15}$$

is an approximate solution of (14). It is difficult to subscribe to the considerations by which they arrive at (15).

It is easy to verify by substitution that an exact solution of (14) is

$$\mathscr{L}(x_0) = \frac{C}{x_0^2}\int_{x_0}^{2x_0} l(\xi)\, d\xi = \frac{C}{x_0^2}\left\{L_-(x_0) - L_-(2x_0)\right\}, \tag{16}$$

where C is a constant and the relation between l and λ is given by (11). Since $\mathscr{L}$ is a frequency function

$$\begin{aligned} 1 &= \int_0^\infty \mathscr{L}(x_0)\, dx_0 \\ &= C\left[\frac{L_-(x_0) - L_-(2x_0)}{x_0}\right]_0^\infty + C\int_0^\infty \frac{2l(2x_0) - l(x_0)}{x_0}\, dx_0 \end{aligned}$$

on integrating by parts. It is found by differentiation that the first term on the right is zero at the lower limit, and so

$$\begin{aligned} 1 &= 2C\int_0^\infty \frac{l(2x_0)}{2x_0}\, d(2x_0) - C\int_0^\infty \frac{l(x_0)}{x_0}\, dx_0 \\ &= C\int_0^\infty \frac{l(x_0)}{x_0}\, dx_0. \end{aligned} \tag{17}$$

The last integral is the moment of order -1 of $l(x_0)$; thus C is the harmonic mean of the distribution l.

The further extension to unsymmetrical fission is less simple. Koch & Schaechter's analysis can be carried through as before, but the contribution to $d\{x_0^2\mathscr{L}(x_0)\}/dx_0$ by newly formed organisms does not now come only from organisms with $x_t = 2x_0$, but from those whose size at termination is x_0/p; and p takes any value in the range 0 to 1 with frequency $k(p)$. Equation (14) now becomes

$$\frac{d}{dx_0}\left\{x_0^2\mathscr{L}(x_0)\right\} = 2\int_0^1 \left(\frac{x_0}{p}\right)^2 \mathscr{L}\left(\frac{x_0}{p}\right)\frac{\lambda(x_0/p)}{\Lambda_-(x_0/p)}\, k(p)\, dp - x_0^2\mathscr{L}(x_0)\frac{\lambda(x_0)}{\Lambda_-(x_0)}. \tag{18}$$

If we take

$$x_0^2\mathscr{L}(x_0) = L_-(x_0) - \int_0^1 k(p)\, L_-(x_0/p)\, dp \tag{19}$$

as a trial solution of (18) (by analogy with (16)) we obtain after reduction

$$\int_0^1 \frac{k(p)}{p} l\left(\frac{x_0}{p}\right) dp = 2\int_0^1 k(p)\, l\left(\frac{x_0}{p}\right) dp,$$

which is in general false. The two integrals have nearly the same value, since p rarely differs much from $\frac{1}{2}$, but (19) is not exact. An exact solution of (18) is derived from Collins & Richmond's (1962) analysis in the next section but one.

Real and artificial distributions

It is convenient at this point to introduce an excursus on the relation between conceptual distributions of generation time or size and the distributions naturally presented by a steadily growing culture. The considerations which I now advance have not, I think, been made explicit elsewhere in the bacteriological literature; they also constitute a justification for the multiplicity of distribution functions which have to be handled in discussing the pattern of growth.

The idea of a distribution of generation times $f(\tau)$ is so familiar as to seem simple. It is not so. Suppose first that generation times are independent of one another, as in Kendall's or Rahn's hypothesis. We can obtain a sample of τ by fixing our attention on a group of fissions selected in any way and recording the τ of *all* the organisms stemming immediately from those fissions. Repetition of the process generates the frequency function $f(\tau)$. But the generation times of the organisms which have, at a given time, completed their life span during the previous history of the culture do not compose $f(\tau)$; they compose the carrier distribution $\mathscr{C}(\tau)$ (Powell, 1956):

$$\mathscr{C}(\tau) \equiv 2e^{-\nu\tau}f(\tau). \tag{20}$$

The distribution of τ among those organisms extant at a given time is, say, $\mathscr{I}(\tau)$, and

$$\mathscr{I}(\tau) \equiv 2(1-e^{-\nu\tau})f(\tau). \tag{21}$$

(I previously stated, incorrectly (Powell, 1956), that $\mathscr{I}(\tau) \equiv f(\tau)$.) When generation times are not independent of one another (as they are not in the hypothesis of Koch & Schaechter and in fact), it is more difficult to obtain an unbiased sample of the τ. For suppose that the generation time of an organism depends on that of its mother. The number of fissions, occurring at any given time, which are the terminations of organisms of generation time τ is proportional to $e^{-\nu\tau}$; at the inception of these organisms the number in the culture, and therefore the absolute fission rate, was less by this factor. (In a steady state continuous culture terminations of organisms of large τ are similarly rare, because the probability of the organisms' remaining in the vessel until termination is also $e^{-\nu\tau}$.) It is not enough, then, to study the organisms stemming from fissions selected apparently at random, because the τ thus made available will be influenced by the τ of the mothers. We may obtain a representative sample by agreeing in advance to record the τ of all the nth generation progeny of selected organisms, n being large enough for these τ to be uninfluenced by the original selection. (In practice, $n = 2$ is probably sufficient to secure a close approximation to independence.) The carrier distribution is no longer

given by (20); organisms whose τ belongs to it must be such that their progeny, after allowance for increase in numbers (or for washout, in continuous cultures) themselves regenerate the carrier distribution. We imagine a 'population' distribution $\mathscr{P}(\tau)$ such that

$$\mathscr{C}(\tau) \equiv 2\, e^{-\nu\tau}\, \mathscr{P}(\tau).$$

Then $\mathscr{P}(\tau)$ is given in terms of $f(\tau)$ and the joint distribution of mother and daughter generation times by means of an integral equation (Powell, 1956). The culture behaves as if the generation times were independent of one another and had the distribution $\mathscr{P}(\tau)$; $\mathscr{P}(\tau)$ replaces $f(\tau)$ in expressions for the carrier and age distributions and for $\mathscr{I}(\tau)$ (equation (21)). The relation between $\mathscr{C}(\tau)$, $\mathscr{P}(\tau)$ and $f(\tau)$ is perhaps most easily seen by considering an imaginary continuous culture with a steady flow of liquid through it. Suppose that, every time a fission occurred, one of the new organisms was selected at random (i.e. without reference to its then unknown generation time) and allowed to leave the culture. The organisms in the vessel would remain constant in number and their generation time distribution would be $f(\tau)$. In a real culture, removal from the vessel depends upon generation time. Those organisms which remain to complete their life span in the vessel have the distribution $\mathscr{C}(\tau)$: those which so remain, together with those which are washed out before termination, have the distribution $\mathscr{P}(\tau)$.

In Koch & Schaechter's hypothesis, $l(x_t)$ corresponds to $f(\tau)$, and we have now to introduce distributions, say $l_C(x_t)$ and $l_P(x_t)$ corresponding to $\mathscr{C}(\tau)$ and $\mathscr{P}(\tau)$. For example, those organisms whose generation times compose $\mathscr{C}(\tau)$ are those whose sizes at termination compose $l_C(x_t)$.

Collins & Richmond's analysis

Collins & Richmond (1962) derived an equation connecting the mean rate of growth of individual organisms (V_x) with the distributions of size at inception and termination and the instantaneous distribution of size at any one time. In its original form it reads

$$Vx = k\left\{2\int_0^{l_x}\Psi(l)\,dl - \int_0^{l_x}\Phi(l)\,dl - \int_0^{l_x}\lambda(l)\,dl\right\}\Big/\lambda(l_x).$$

Collins & Richmond's λ is our $\mathscr{L}$, their k is our ν, their l_x is our x_0 and, in Koch & Schaechter's hypothesis, Vx is simply νx, since $d\log x/dt = \nu$. We can write, retaining Ψ and Φ for the moment,

$$x_0\mathscr{L}(x_0) = 2\int_0^{x_0}\Psi(\xi)\,d\xi - \int_0^{x_0}\Phi(\xi)\,d\xi - \int_0^{x_0}\mathscr{L}(\xi)\,d\xi.$$

On differentiating, we obtain a differential equation easily soluble for $\mathscr{L}$:

$$x_0\frac{d\mathscr{L}}{dx_0} + 2\mathscr{L} = 2\Psi - \Phi$$

and

$$\mathscr{L}(x_0) = \frac{1}{x_0^2}\int_0^{x_0}\xi\{2\Psi(\xi) - \Phi(\xi)\}\,d\xi. \tag{22}$$

If fission is symmetrical,

$$\mathscr{L}(x_0) = \frac{1}{x_0^2}\int_0^{x_0}\xi\{4\Phi(2\xi) - \Phi(\xi)\}\,d\xi \tag{23}$$

$$= \frac{1}{x_0^2}\int_{x_0}^{2x_0}\xi\,\Phi(\xi)\,d\xi. \tag{24}$$

Collins & Richmond's distribution of size at termination, $\Phi(x_t)$, is not our $l(x_t)$; it is the distribution $l_C(x_t)$ of the size at termination of those organisms whose τ belong to $\mathscr{C}(\tau)$ (compare (24) with (16)). It follows that when fission is symmetrical

$$l_C(x_t) = Cl(x_t)/x_t.$$

More generally when fission is not symmetrical, the distribution of size at inception actually realized up to a given time in a growing culture is

$$\int_0^1 \frac{k(p)}{p} l_C\left(\frac{x_i}{p}\right) dp,$$

and (22) becomes

$$\mathscr{L}(x_0) = \frac{1}{x_0^2}\left\{2\int_0^{x_0}\int_0^1 \xi l_C\left(\frac{\xi}{p}\right)\frac{k(p)}{p}\,dp\,d\xi - \int_0^{x_0}\xi l_C(\xi)\,d\xi\right\}. \tag{25}$$

We can obtain still another relation between $\mathscr{L}(x_0)$ and $l_C(x_t)$. When the number in the culture is N, there are $N\mathscr{L}(x_0)\,dx_0$ organisms whose size is in the range x_0, x_0+dx_0. During a short time dt there are $N\nu\,dt$ fissions which are the termination of organisms belonging to $l_C(x_t)$. In the same interval organisms of size x_0 would grow, in the absence of fission, from x_0 to $x_0\,e^{\nu dt}$, $\doteq x_0(1+\nu\,dt)$—an increment of $x_0\nu\,dt$. The number which divide is thus

$$N\mathscr{L}(x_0)\,dx_0\frac{\lambda(x_0)}{\Lambda_-(x_0)}x_0\nu\,dt.$$

But the number dividing in the size range x_0, x_0+dx_0 is also $Nl_C(x_0)\,dx_0\nu\,dt$ and so

$$l_C(x_t) = \mathscr{L}(x_t)\frac{x_t\lambda(x_t)}{\Lambda_-(x_t)}. \tag{26}$$

It is easily found by substitution that (25) and (26) are together consistent with the general form of Koch & Schaechter's equation (18). Also they give a relation between λ and l_C analogous to (13):

$$\frac{\lambda(x_t)}{\Lambda_-(x_t)} = x_t l_C(x_t)\Big/\left\{2\int_0^{x_t}\int_0^1 \xi\frac{k(p)}{p}l_C\left(\frac{\xi}{p}\right)dp\,d\xi - \int_0^{x_t}\xi l_C(\xi)\,d\xi\right\}. \tag{27}$$

Approximate distributions of size and generation time

It is now easy to elaborate a series of varied and complex relations between λ, l, l_C, l_P, $\mathscr{L}$, f, $\mathscr{C}$ and $\mathscr{P}$. Most of them are beyond experimental test for want of data sufficiently numerous and precise. Moreover, no hypothesis about the pattern of growth is likely to agree more than broadly with the known phenomena. It is therefore useless to pursue mathematical exactitude; instead we should seek some approximation which will make algebraic manipulation simpler and such that its magnitude can be estimated experimentally.

A simplifying approximation

Let us suppose that every organism passes through a size X at some time during its life span. This implies that the two distributions l_i, l do not overlap; i.e. the largest x_i ever to occur is not greater than the smallest x_t: X lies between these extremes. (It will be convenient in the sequel to take X to be equal to or just less than the smallest x_t.) It implies further a restriction on the range of p: px_t must never be greater than X.

On the assumption that the distribution of p is independent of x_t we can suppose its range to be say $\frac{1}{2} \pm \epsilon$. Then the range of x_t must be from X to $X/(\frac{1}{2}+\epsilon)$ and of x_i from $X(\frac{1}{2}-\epsilon)$ to X. The range of the joint distribution of x_i and x_t is illustrated in Fig. 3. The corresponding range of generation times will be from $\tau = 0$ (when

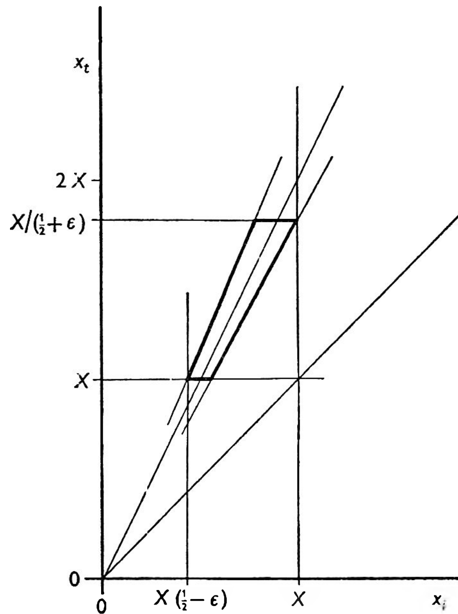


Fig. 3. When the distributions of x_i and x_t do not overlap, the joint distribution is confined to the interior of a trapezium (heavy line). Its limits are given in terms of the critical size X and the range $\frac{1}{2} \pm \epsilon$ of the ratio p.

for the same organism $x_i = X = x_t$) to a little more than $2\bar{\tau}$ (when $x_i = X(\frac{1}{2}-\epsilon)$ and $x_t = X/(\frac{1}{2}+\epsilon)$, and $\tau = \log(x_t/x_i)/\nu \simeq 2\bar{\tau}$). In particular, $l_i(x_i)$ will be zero for $x_i > X$ and so in equations (10) and (13) the second term in the denominator on the right will vanish for all x_t for which $l(x_t)$ is not 0.

Under this simplifying assumption, then, we have

$$\lambda(x_t) \equiv l(x_t); \tag{28}$$

that is, the realized distribution of length at fission is identical with Koch & Schaechter's intrinsic distribution $\lambda(x_t)$. Koch & Schaechter based their treatment on the assumption that the coefficient of variation of x_t was small; in fact it is the limited range which is the necessary condition for the truth of their inferences; the only logical restriction on the coefficient of variation is then that it should be less than $1/\sqrt{8} \simeq 0{\cdot}35$.

The distribution of generation times

Under the simplifying assumption we have just made, equation (3) represents the distribution of τ when fission results in daughters of equal size. More generally, the factor $2l(2x_i)$ in (2) has to be replaced by (12), and

$$f(\tau) = \nu \int_0^\infty \int_0^1 x_i\, e^{\nu\tau} \frac{k(p)}{p}\, l\left(\frac{x_i}{p}\right) l(x_i\, e^{\nu\tau})\, dp\, dx_i, \tag{29}$$

where the subscript s of f has been dropped since this frequency function is not symmetrical. It has now to be remembered that the integrand in (29) is zero when the argument of either of the l-functions lies outside the range X, $2X$; the effective limits of the integral (29) are

$$x_i(\tfrac{1}{2}+\epsilon)/X < p < x_i/X \quad \text{and} \quad X\,e^{-\nu\tau} < x_i < X\,e^{-\nu\tau}/(\tfrac{1}{2}-\epsilon).$$

The mean of the distribution is

$$\bar{\tau} = \nu \int\!\!\int\!\!\int \tau\, e^{\nu\tau} \frac{k(p)}{p}\, l\left(\frac{x_i}{p}\right) l(x_i\, e^{\nu\tau})\, x_i\, dp\, dx_i\, d\tau,$$

the integration being carried over the whole range for which l and k are not 0. On putting $x_t = x_i/p$ and $\xi = x_i\, e^{\nu\tau}$ it becomes

$$\begin{aligned}\bar{\tau} &= \frac{1}{\nu}\int\!\!\int\!\!\int (\log\xi - \log x_t - \log p)\, k(p)\, l(x_t)\, l(\xi)\, dp\, dx_t\, d\xi \\ &= -\frac{1}{\nu}\int (\log p)\, k(p)\, dp \\ &= -\mu_1'(\log p)/\nu. \end{aligned} \tag{30}$$

The geometric mean of p, namely $\exp\{\mu_1'(\log p)\}$, is by a general rule less than its arithmetic mean ($\frac{1}{2}$), hence $-\mu_1'(\log p) > \log 2$: for a given ν, $\bar{\tau}$ is increased by the fission of organisms into daughters of unequal size.

The other moments about the origin are given by

$$\mu_r'(\tau) = \nu \int\!\!\int\!\!\int \tau^r\, e^{\nu\tau} \frac{k(p)}{p}\, l\left(\frac{x_i}{p}\right) l(x_i\, e^{\nu\tau})\, x_i\, dp\, dx_i\, d\tau.$$

The same substitution as before yields

$$\mu_r'(\tau) = \nu^{-r} \int\!\!\int\!\!\int (\log\xi - \log x_t - \log p)^r\, k(p)\, l(x_t)\, l(\xi)\, dp\, dx_t\, d\xi.$$

This expression can be dealt with expeditiously by noting that the right-hand side is the rth derivative with respect to s of

$$\int\!\!\int\!\!\int (\xi/px_t)^{(s-1)/\nu}\, k(p)\, l(x_t)\, l(\xi)\, dp\, dx_t\, d\xi,$$

when $s = 1$. The factor $(\xi/px_t)^{(s-1)/\nu}$ can be written

$$\xi^{(s+\nu-1)/\nu-1} \,.\, p^{(\nu+1-s)/\nu-1} \,.\, x_t^{(\nu+1-s)/\nu-1},$$

and so

$$\mu_r'(\tau) = \frac{d^r}{ds^r}\left\{\mathfrak{M}\left(\frac{\nu+s-1}{\nu}; l\right) \mathfrak{M}\left(\frac{\nu+1-s}{\nu}; l\right) \mathfrak{M}\left(\frac{\nu+1-s}{\nu}; k\right)\right\}\Bigg|_{s=1}, \tag{31}$$

where $\mathfrak{M}(s; l)$, $\mathfrak{M}(s; k)$ are respectively the Mellin transforms of $l(x_t)$ and $k(p)$. Since the operand in (31) generates the moments, its logarithm generates the cumulants:

$$\kappa_r(\tau) = \frac{d^r}{ds^r}\left[\log\left\{\mathfrak{M}\left(\frac{\nu+s-1}{\nu}; l\right) \mathfrak{M}\left(\frac{\nu+1-s}{\nu}; l\right) \mathfrak{M}\left(\frac{\nu+1-s}{\nu}; k\right)\right\}\right]\Bigg|_{s=1}. \tag{32}$$

In particular

$$\operatorname{var}\tau = \kappa_2(\tau) = \nu^{-2}(2\operatorname{var}\log x_t + \operatorname{var}\log p), \tag{33}$$

or writing c for a coefficient of variation, and putting $\nu = -\mu_1'(\log p)/\bar{\tau}$,

$$c^2(\tau) \simeq \{2c^2(x_t) + c^2(p)\}/\{\mu_1'(\log p)\}^2.$$

When p is not appreciably dispersed,

$$c^2(p) \ll c^2(x_t) \quad \text{and} \quad \mu_1'(\log p) \simeq \log \tfrac{1}{2}; \quad (\log \tfrac{1}{2})^2 \simeq \tfrac{1}{2},$$

so

$$c^2(\tau) \simeq 4c^2(x_t).$$

Thus $c(\tau)$ is about twice $c(x_t)$. Schaechter *et al.* (1962) found this to be roughly true of the organisms they examined.

The third moment of $f(\tau)$, from (32), is

$$\mu_3(\tau) = \kappa_3(\tau) = -\mu_3(\log p)/\nu^3,$$

and from this we find that the skewness of $f(\tau)$ is

$$\gamma_1(\tau) = \mu_3(\tau)/\{\mu_2(\tau)\}^{\frac{3}{2}} = \frac{-\mu_3(\log p)}{\{-\mu_1'(\log p)\}^3\, c^3(\tau)};$$

this is a positive number since the expectations of odd powers of $\log p$ are all negative. As Koch & Schaechter suggested, lack of symmetry at fission introduces positive skewness into $f(\tau)$.

The application of an integral transform gives us a general method of handling (29), particularly when specific frequency functions are involved, but the simple results (30) and (33) can be obtained more readily by a direct appeal to the equation

$$\tau = \frac{1}{\nu}\log\left(\frac{x_t'}{x_i}\right),$$

in which it is to be understood that x_i and x_t' belong to the same organism, not the same fission.

The carrier distributions

The simplifying assumption implies that the size of an organism at termination is statistically as well as biologically independent of its size at inception. Since each organism passes through the size X, its contributions to $l_C(x_t)$, $l_D(x_t)$, $l(x_t)$ can only differ because of its history after reaching X. We can say at once that $l_C(x_t)$ is proportional to $l(x_t)\, e^{-\nu t_X}$, where t_X is the time that elapses between the attainment of size X and termination. But by the original postulates

$$x_t = X\, e^{\nu t}$$

and so

$$l_C(x_t) \propto l(x_t)/x_t.$$

The constant of proportionality is evidently the harmonic mean, C, of the distribution $l(x_t)$ (equation 17). This accords with (27); obviously l_C and l have the same range, and the first integral in the denominator of (27) covers the whole range of $l_C(\xi/p)$ for any x_t for which $l_C(x_t)$ is not zero. This term becomes

$$2\int_0^1\int_0^{x_t} p k(p)\frac{\xi}{p}\, l_C\left(\frac{\xi}{p}\right)\frac{d\xi}{p}\, dp = 2C\int_0^1 p\, k(p)\, dp$$
$$= C;$$

$$C - \int_0^{x_t} \xi\, l_C(\xi)\, d\xi = \int_{x_t}^{\infty} \xi\, l_C(\xi)\, d\xi,$$

and

$$\frac{x_t l_C(x_t)}{\int_{x_t}^{\infty} \xi\, l_C(\xi)\, d\xi} = \frac{\lambda(x_t)}{\Lambda_-(x_t)} = \frac{l(x_t)}{L_-(x_t)}.$$

Thus we now have, whatever the dispersion of p,

$$l_C(x_t) \equiv Cl(x_t)/x_t. \tag{34}$$

The carrier distribution of generation time, $\mathscr{C}(\tau)$, can now be written down at once, by comparison with (29):

$$\begin{aligned}\mathscr{C}(\tau) &= \nu \int_0^\infty \int_0^1 \frac{k(p)}{p}\, l_C\left(\frac{x_i}{p}\right) l_C(x_i\, e^{\nu\tau})\, x_i\, e^{\nu\tau}\, dp\, dx_i \\ &= C^2\nu \int_0^\infty \int_0^1 \frac{k(p)}{x_i}\, l\left(\frac{x_i}{p}\right) l(x_i\, e^{\nu\tau})\, dp\, dx_i.\end{aligned}$$

It is clear that under the simplifying assumption $l_P(x_t)$, corresponding to $\mathscr{P}(\tau)$, is identical with $l(x_t)$, because now each x_t is independent of the preceding x_i. But it is not true that $\mathscr{P}(\tau) \equiv f(\tau)$ because the τ depend on the x_i as well as the x_t. A special investigation is not necessary; since

$$2e^{-\nu\tau}\mathscr{P}(\tau) \equiv \mathscr{C}(\tau),$$

we have

$$\mathscr{P}(\tau) = \frac{C^2\nu\, e^{\nu\tau}}{2} \int_0^\infty \int_0^1 \frac{k(p)}{x_i}\, l\left(\frac{x_i}{p}\right) l(x_i\, e^{\nu\tau})\, dp\, dx_i. \tag{35}$$

The frequency function of age, $\phi(a)$, is given by

$$\phi(a) = 2\nu\, e^{-\nu a} \int_a^\infty \mathscr{P}(\tau)\, d\tau;$$

on replacing $\mathscr{P}(\tau)$ from (35) and changing the order of integration, it becomes

$$\phi(a) = C^2\nu\, e^{-\nu a} \int_0^\infty \int_0^1 \frac{k(p)}{x_i}\, l\left(\frac{x_i}{p}\right) L_-(x_i\, e^{\nu a})\, dp\, dx_i.$$

Correlations between generation times

The principal correlations which have to be accounted for by any hypothesis are those between the generation times of mothers and daughters, and between the generation times of sisters.

Suppose we have an organism of generation time τ_m whose extremes of size are x', x'_t. Let the generation time of one of its daughters be τ_d and let the extreme sizes of this daughter be x_i, x_t. Then x_i is a fraction p of x'_t and

$$x_t = x_i \exp\{\nu\tau_d\} = px'_t \exp\{\nu\tau_d\} = px'_i \exp\{\nu(\tau_d+\tau_m)\},$$

i.e.

$$\tau_d+\tau_m = \frac{1}{\nu}\log\left(\frac{x_t}{x'_i p}\right).$$

Then

$$\operatorname{var}(\tau_d+\tau_m) = \nu^{-2}(\operatorname{var}\log x_t + \operatorname{var}\log x'_i + \operatorname{var}\log p);$$

under the assumptions that p is independent of the x_t with which it is associated and that the distributions $l(x_t)$, $l_i(x_i)$ do not overlap, the covariances between x_t, x'_i and p are all zero. We have now to relate var log x'_i to var log x_t; since these statistics are characteristics of functions $l_i(x_i)$, $l(x_t)$, the prime is irrelevant. Every x_i belongs to the same fission as some x_t, and then

$$x_i = px_t;$$

$$\operatorname{var}\log x_i = \operatorname{var}\log p + \operatorname{var}\log x_t.$$

Hence

$$\operatorname{var}(\tau_d+\tau_m) = 2\nu^{-2}(\operatorname{var}\log x_t + \operatorname{var}\log p).$$

But $\rho(H)$, the product-moment correlation between the generation times of mothers and daughters, is given by

$$\rho(H) = \frac{\text{var}(\tau_d+\tau_m)}{2\,\text{var}\,\tau} - 1,$$

and we find, using (33),

$$\rho(H) = \frac{-\text{var}\log x_t}{2\,\text{var}\log x_t + \text{var}\log p}. \tag{36}$$

The corresponding analysis for sisters is a little more complicated. If τ_1, τ_2 are the generation times of a pair of sisters whose terminal sizes are x_t', x_t'', we have

$$\tau_1 = \frac{1}{\nu}\log\left(\frac{x_t'}{px_t}\right); \quad \tau_2 = \frac{1}{\nu}\log\left\{\frac{x_t''}{(1-p)\,x_t}\right\},$$

where x_t is the terminal size of the mother. Then

$$\tau_1-\tau_2 = \frac{1}{\nu}\log\left\{\frac{x_t'(1-p)}{px_t''}\right\};$$

$$\text{var}(\tau_1-\tau_2) = \nu^{-2}[2\,\text{var}\log x_t + \text{var}\log\{(1-p)/p\}].$$

By symmetry, $\text{var}\log(1-p) = \text{var}\log p$, but here p and $1-p$ are algebraically related, not separate realizations of the variate p. Writing E for expectation, we have

$$\text{var}\log p = E(\log p)^2 - \{E(\log p)\}^2$$

$$\text{var}\log\{(1-p)/p\} = 2E(\log p)^2 - 2E\{\log p.\log(1-p)\};$$

also

$$\text{var}\log\{p(1-p)\} = 2E(\log p)^2 + 2E\{\log p\log(1-p)\} - 4\{E(\log p)\}^2.$$

So

$$\text{var}\log\{(1-p)/p\} + \text{var}\log\{p(1-p)\} = 4E(\log p)^2 - 4\{E(\log p)\}^2 = 4\,\text{var}\log p,$$

and

$$\text{var}(\tau_1-\tau_2) = \nu^{-2}[2\,\text{var}\log x_t + 4\,\text{var}\log p - \text{var}\log\{p(1-p)\}].$$

The expression $p(1-p)$, *qua* function of p, has a maximum at $p = \frac{1}{2}$; it therefore changes little over the important range: the observed p are closely grouped about $p = \frac{1}{2}$. Thus $\text{var}\log\{p(1-p)\}$ is relatively small. If we omit this term, we have, very nearly,

$$\rho(S) = 1 - \frac{\text{var}(\tau_1-\tau_2)}{2\,\text{var}\,\tau}$$

$$= \frac{\text{var}\log x_t - \text{var}\log p}{2\,\text{var}\log x_t + \text{var}\log p}. \tag{37}$$

Koch & Schaechter's approximate formulae, in which coefficients of variation replace variances of logarithms, agree with (36) and (37).

When the dispersion of p is small enough, then,

$$-\rho(H) = \rho(S) = \tfrac{1}{2}.$$

Koch & Schaechter's hypothesis is an advance on all previous ideas in that it predicts that $\rho(H)$ and $\rho(S)$ will not be negligible and will be respectively negative and positive. It does not account for the deeper hereditary properties evinced as correlations between the τ of cousins and second cousins, nor does it account

quantitatively for the divergence of $-\rho(H)$, $\rho(S)$ from $\frac{1}{2}$. According to (36) and (37), the divergences result from the dispersion of p, and

$$\rho(S) \leqslant -\rho(H) \leqslant \tfrac{1}{2};$$
$$\rho(S)+\rho(H) \leqslant 0.$$

In practice the inequalities are not found to be true; with every allowance for experimental imperfection, $\rho(S)$ usually exceeds $\frac{1}{2}$ and $-\rho(H)$ rarely attains it (Powell, 1958; Powell & Errington, 1963*a*; Schaechter *et al.* 1962).

Justification of the assumptions

It is not the purpose of this note to attempt a general critique of the Koch & Schaechter hypothesis, but only to suggest how, by a mathematical simplification, its consequences can best be compared with experiment. We arrive at manageable formulae by making two special assumptions, but we cannot fairly use these formulae to test the hypothesis unless the assumptions which make them valid are true (or very nearly true) of the data to which they are to be applied. Accordingly I now adduce some evidence that the assumptions are often true of real cultures.

The first and probably much the less important assumption is that inequality in the size of sister organisms at inception is independent of the size of the mother at termination; more precisely, that the distribution of the parameter p is the same for all x_t. There is as yet no direct evidence bearing on this point.

The second assumption is that the distributions of length at inception and length at termination do not overlap or, what is logically equivalent, that there is a size X which is attained by every organism during its life span. A further implication is that p and x_t are jointly restricted so that $px_t \leqslant X$ for all p, and we can suppose that the range of p is $\frac{1}{2} \pm \epsilon$. It now follows that the possible extremes of x_i are X, $X(\frac{1}{2}-\epsilon)$—a range of a little more than 2:1; for x_t they are X, $X/(\frac{1}{2}+\epsilon)$—a range of a little less than 2:1. Further, the possible extremes of x_0, the cell size in the steady-state population, are $X(\frac{1}{2}-\epsilon)$, $X/(\frac{1}{2}+\epsilon)$—a range of a little more than 4:1.

The measurements of Collins & Richmond (1962) on *Bacillus cereus* provide an interesting example. Within the accuracy of their technique, they found the ratio of the extremes of x_0 to be just 4:1. This result is perhaps unusual, since a much wider range of x_0 is common in the *Bacillus* group, and indeed we need not expect the Koch & Schaechter hypothesis, still less our restrictive assumptions, to apply directly to whole multicellular organisms.

Powell & Errington (1963*b*) measured the volumes of *Aerobacter aerogenes* (Hinshelwood strain) growing in a defined medium at two temperatures. The growth rate was limited to 0·2 hr^{-1} in a continuous culture. The ratios of extreme volumes in the two series of measurements were 4·0:1, 3·9:1. On the other hand, Dr J. R. Postgate (private communication) isolated a variant of the Hinshelwood strain which was apparently stable under similar conditions, when Powell & Errington (1963*b*) found the ratio of its extreme volumes to be 12·5:1. It seems likely, however, that the restrictive conditions of continuous culture tend to make for uniformity of size. This can be true of so unpromising a species as *Proteus vulgaris*. Powell & Errington (unpublished) grew the organism in continuous culture on a chemically defined medium ('H12P*'; Powell & Errington, 1963*a*) at growth rates of 0·28 and 0·62 hr^{-1}. The corresponding ratios of extreme volumes were 3·3:1, 2·8:1.

Errington, Powell & Thompson (unpublished) measured the lengths at inception (x_i) and termination (x_t) of *Escherichia coli*, *Pseudomonas aeruginosa* and *Serratia marcescens* in four groups of experiments. In each of three groups, the ranges of x_i and x_t were less than 2:1, the greatest x_t was less than four times the least x_i and the least x_t was greater than the greatest x_i. In the fourth group, the limits were exceeded only by the x_i and x_t associated with a single fission.

Apart from those of Collins & Richmond, the above data were obtained from rather small samples (about 100 organisms). It is to be remembered that the expected range of a sample is less than that of the population from which it is drawn by an amount which is greater, the smaller the sample size.

Further evidence can be obtained from an examination of the range of generation times, τ. Under the Koch & Schaechter hypothesis and the simplifying assumptions,

$$\begin{aligned} 0 \leqslant \tau &\leqslant \frac{1}{\nu} \log \left\{ \frac{X/(\frac{1}{2}+\epsilon)}{X(\frac{1}{2}-\epsilon)} \right\} \\ &= -\nu^{-1} \log(\tfrac{1}{4}-\epsilon^2) \\ &= -2\nu^{-1} \log\{(\tfrac{1}{4}-\epsilon^2)^{\frac{1}{2}}\}. \end{aligned}$$

Now, by (30), $\nu = -\mu_1'(\log p)/\bar{\tau}$, so

$$0 \leqslant \tau \leqslant 2\bar{\tau} \frac{-\log\{(\frac{1}{4}-\epsilon^2)^{\frac{1}{2}}\}}{-\mu_1'(\log p)}. \tag{38}$$

But, since the distribution of p is closely concentrated round the value $\frac{1}{2}$, the geometric mean of p is greater than the geometric mean of its extremes, $(\frac{1}{4}-\epsilon^2)^{\frac{1}{2}}$. That is

$$\begin{aligned} \exp\{\mu_1'(\log p)\} &> (\tfrac{1}{4}-\epsilon^2)^{\frac{1}{2}}; \\ \mu_1'(\log p) &> \log\{(\tfrac{1}{4}-\epsilon^2)^{\frac{1}{2}}\}. \end{aligned}$$

Both members of the last inequality are negative, hence the last member of (38) is greater than $2\bar{\tau}$: the admissible range of τ is from 0 to a little more than $2\bar{\tau}$. Information about the range of τ in real cultures is less cogent than information about the range of size, because (38) is a necessary but not sufficient condition for the truth of the simplifying assumption; however, there is a great deal more of it.

The inequality

$$0 \leqslant \tau \leqslant 2\bar{\tau}$$

is evidently satisfied by the examples in Schaechter *et al.* (1962). Powell (1958) measured in all about 6000 generation times in 16 groups of experiments. Reference to the original records showed that, omitting non-viable organisms, only 7 of the individual τ exceeded twice the mean for the group to which they belonged; in the least favourable group, 2 of 367 generation times did not satisfy the inequality. Powell & Errington's (1963*a*) data gave a similar ratio of 8 exceptions among 5000; at worst 4 in a group of 963 measurements.

Thus the required restrictions on the range of x_i, x_t, x_0 and τ are most strikingly met, to within a little, by many cultures of Gram-negative organisms.

The critical size

We introduced a critical size X in the first place to simplify the mathematical relationships implied by the Koch & Schaechter hypothesis. It is remarkable that real cultures so nearly satisfy the conditions which make the simplified formulae

valid. We might perhaps identify X with Koch & Schaechter's critical size $\bar{c}$; it is not essential to the hypothesis that $\bar{c}$ should in any sense be a mean of the observed size of organisms at termination. It will be, as I think, at least temporarily profitable to consider the relation of X to a more fundamental critical size which we may call Ξ: this is the absolute minimum size at which an organism of a given species can divide into two viable daughters; a size determined by the minimum complement of enzymes, nuclear material and mechanical structural elements. The size Ξ may be attained under the starvation conditions of a continuous culture run at a low dilution rate (D. Herbert & D. W. Tempest, private communication; see also Herbert, 1958); under less arduous conditions we can expect the lower observed limit, X, to be larger than Ξ and less clearly defined.

We can then, following Koch & Schaechter, suppose that fission succeeds the attainment of the size X after a variable interval determined by secondary causes. That the size $2X$ is rarely exceeded means that organisms which fail to divide before four nuclei have developed are themselves rare, as the experiments of Schaechter *et al.* (1962) suggest.

REFERENCES

Adolph, E. F. & Bayne-Jones, S. (1932). Growth in size of micro-organisms measured from motion pictures. II. *Bacillus megatherium*. *J. cell. comp. Physiol.* **1**, 409.

Bayne-Jones, S. & Adolph, E. F. (1932). Growth in size of micro-organisms measured from motion pictures. III. *Bacterium coli*. *J. cell. comp. Physiol.* **2**, 329.

Collins, J. F. & Richmond, M. H. (1962). Rate of growth of *Bacillus cereus* between divisions. *J. gen. Microbiol.* **28**, 15.

Erdélyi, A. (1954). *Tables of Integral Transforms*, vol. I. New York: McGraw-Hill Book Co. Inc.

Herbert, D. (1958). Some principles of continuous culture. In *Recent Progress in Microbiology*. Ed. Tunevall, G. Stockholm: Almqvist and Wiksell.

Kendall, D. G. (1948). On the role of a variable generation time in the development of a stochastic birth process. *Biometrika*, **35**, 316.

Koch, A. L. & Schaechter, M. (1962). A model for statistics of the cell division process. *J. gen. Microbiol.* **29**, 435.

Powell, E. O. (1955). Some features of the generation times of individual bacteria. *Biometrika*, **42**, 16.

Powell, E. O. (1956). Growth rate and generation time of bacteria, with special reference to continuous culture. *J. gen. Microbiol.* **15**, 492.

Powell, E. O. (1958). An outline of the pattern of bacterial generation times. *J. gen. Microbiol.* **18**, 382.

Powell, E. O. & Errington, F. P. (1963*a*). Generation times of individual bacteria: some corroborative measurements. *J. gen. Microbiol.* **31**, 315.

Powell, E. O. & Errington, F. P. (1963*b*). The size of bacteria, as measured with the Dyson image-splitting eyepiece. *J. R. micr. Soc.* **82**, 39.

Rahn, O. (1932). A chemical explanation of the variability of the growth-rate. *J. gen. Physiol.* **15**, 257.

Schaechter, M., Williamson, J. P., Hood, J. R. & Koch, A. L. (1962). Growth, cell and nuclear divisions in some bacteria. *J. gen. Microbiol.* **29**, 421.

J. gen. Microbiol. (1964), 37, 251–258
Printed in Great Britain

Studies on the Heat Stability and Chromatographic Behaviour of the Scrapie Agent

BY G. D. HUNTER AND G. C. MILLSON

Agricultural Research Council, Institute for Research on Animal Diseases, Compton, Newbury, Berkshire

(*Received* 9 *June* 1964)

SUMMARY

Saline extracts of mouse-scrapie brain homogenates lost little infectivity when heated at 75° for one hour. At higher temperatures, there was a progressive inactivation of the agent, although some of the infectivity still remained after heating at 100° for one hour. The smooth shape of the inactivation curve suggests that the scrapie agent exists predominantly in a single heat-stable form. Ultrasonic disruption of scrapie mitochondrial suspensions altered the chromatographic behaviour of the scrapie agent in a way which suggests that the free agent is relatively small. The agent associates very readily with denatured proteinaceous material and because of this property it has not yet been possible to separate it effectively from contaminating tissue debris.

INTRODUCTION

Previous experiments with the scrapie agent passaged in mice (Hunter, Millson & Chandler, 1963) showed that the bulk of the agent in brain homogenates was associated with the particulate fractions of the cells. The highest infectivity was found in the mitochondrial-lysosomal fraction (Hunter, Millson & Meek, 1964), although it was pointed out (Mould, Dawson & Smith, 1964) that the concentration of the agent in the microsomal fractions of the brain cells was almost as great. None of these studies gave any clear indication of the nature of the association of the scrapie agent with the particulate fractions of the brain cells. Evidence is presented in this paper to show that the scrapie agent is not in fact comparable in size and density with mitochondria or lysosomes, but is a smaller agent that binds very firmly to tissue debris.

The experiments of Chandler (1963) confirmed to some extent that the mouse-passaged scrapie agent was as resistant to chemical and physical attack as the sheep and goat agent had earlier been found to be (see review by Stamp, 1962). None of this earlier work, however, was of a quantitative nature, and the resistance of the agent to heat in particular was reminiscent of the behaviour of bacterial spores. It seemed possible that the agent might exist in more than one form, only one of which was heat resistant. However, the evidence presented in this paper suggests that the scrapie agent exists in a single heat-stable form.

METHODS

Animal experiments. B.S.V.S. (bacterial susceptible, virus susceptible; Schneider, 1959) white mice were used in all experiments. Cases of scrapie were diagnosed as described previously (Hunter *et al.* 1963) following the criteria laid down by Chandler (1963) and by Pattison & Smith (1963). Groups of at least six mice were used in all titration experiments, and all mice were inoculated intracerebrally with 0·02 ml. inoculum. ID50 values, calculated according to the Kärber method (Parker, 1959), were all related back to the weight of the brain from which the relevant material was originally derived.

Application of heat to the scrapie agent. Six scrapie-affected mice were decapitated, the brains removed immediately and homogenized in a Potter-type homogenizer in physiological saline (25 ml.). Readily sedimentable tissue debris was removed by centrifugation at 500g for 10 min. and rejected. A portion of the supernatant fluid (0·5 ml.) was set aside and used for the preparation of saline dilutions as control material. One ml. portions of the supernatant fluid were placed in small tubes which were sealed in a blow-pipe flame. The tubes were heated for either 10 min. or 60 min. in water baths maintained at 50°, 62·5°, 75°, 87·5° or 100°, and in the case of the higher temperatures in baths of boiling n-butanol (118°) or xylene (141°). At the end of the required period, the tubes were removed from the liquid baths with tongs and cooled to room temperature as rapidly as possible. The tubes were cut open and the whole contents used for the preparation of saline dilutions in the usual way. Three dilutions only (10^{-2}, 10^{-3}, 10^{-4}) were prepared for inoculation into mice and the infective titres calculated from the median incubation periods as described previously (Hunter *et al.* 1963).

In a further experiment, a crude scrapie mitochondrial suspension was prepared from the brains of three clinically affected mice as described previously (Hunter *et al.* 1964). This suspension was centrifuged at 40,000g for 30 min., and the pellet containing mitochondria, lysosomes, nerve-ending particles and a little myelin was re-homogenized in 10 ml. saline. This suspension was sealed in a glass tube and heated at 75–80° for 60 min. After cooling and opening the tube, coagulated material was sedimented at 500g for 10 min. A 'mitochondrial' fraction was sedimented from the supernatant fluid by centrifuging at 10,000g for 15 min., and finally a 'microsomal' fraction was prepared from the second supernatant fluid at 100,000g for 90 min. Saline dilutions of all three particulate fractions and of the original saline suspension and the final supernatant 'soluble' fraction were prepared for titration in mice.

Stability of the frozen scrapie agent. Saline dilutions of whole brain homogenates from scrapie affected mice were maintained between −23° and −28° after inoculation into mice. After 9 months had elapsed a second group of mice was inoculated with the same suspensions and the titrations of the scrapie agent in the two groups were compared.

Ultrasonic disintegration of scrapie mitochondrial suspensions. A crude suspension of scrapie mitochondria and lysosomes was prepared as described previously (Hunter *et al.* 1963) and sedimented at 10,000g for 50 min. The pellet was re-homogenized gently in 0·3 M-sucrose (11 ml.), a portion retained for control dilutions, and the rest treated ultrasonically with a 60 W. M.S.E.—Mullard ultrasonic

disintegrator for 20 min., during which it was kept cool (≯ 10°) with an ice and water bath. A portion of the unfractionated ultrasonically treated material was retained for the preparation of saline dilutions, and the remainder was separated into a 'mitochondrial' fraction sedimented at 10,000***g*** for 50 min., a 'microsomal' fraction sedimenting at 100,000***g*** in 120 min., and a final 'soluble' fraction. Saline dilutions of all three fractions were prepared for titration in mice.

Action of a neutral detergent on the scrapie agent. A crude suspension of scrapie brain mitochondria was prepared as above from six scrapie-affected mice. After centrifugation at 10,000***g*** for 15 min., one half of the sediment was suspended in saline and a portion used for the preparation of controls for mouse inoculation. The other half of the sediment was suspended in 0·01 % Tween 80 (supplied by Honeywill and Stein Ltd., London, W.1) in 0·3 M-sucrose (12 ml.), homogenized and maintained at 2° for 1 hr. A precipitate formed and was sedimented at 500***g*** for 10 min. A 'mitochondrial' fraction was collected from the supernatant solution by centrifugation at 10,000***g*** for 15 min., and a 'microsomal' fraction from the further supernatant solution was deposited by 100,000***g*** for 60 min. Saline dilutions of these particulate fractions and of the final 'soluble' supernatant fraction were prepared and titrated in mice.

Chromatography of the scrapie agent. DEAE-cellulose was suspended in water and the fine particles removed by repeated decantation. Columns 1 × 9 cm., prepared and packed under positive pressure (50 mm. Hg), were washed with about 50 ml. 0·01 N-NaOH and finally with 0·01 M-phosphate buffer (pH 7·5) until the effluent became pH 7·5. Purified scrapie mitochondria were prepared as described previously (Hunter *et al.* 1963) and re-homogenized in 0·01 M-phosphate buffer before application to the columns. Material derived from the equivalent of two mouse brains in 9 ml. was applied to each column, and eluted with successive 50 ml. portions of 0·01 M-phosphate buffer, 0·1 M-NaCl, 0·15 M-NaCl, 0·30 M-NaCl, and 1·00 M-NaCl; all the NaCl solutions were buffered with 0·01 M-phosphate buffer (pH 7·5). A portion of the purified scrapie mitochondrial suspension was subjected to ultrasonic treatment for 15 min. before chromatography on DEAE-cellulose, under similar conditions. In one experiment a DEAE-cellulose column was loaded with ultrasonically-disrupted scrapie mitochondria as before, but the material eluted directly with 0·01 M-phosphate buffer was further separated into 'mitochondrial', 'microsomal', and soluble fractions by successive sedimentations at 10,000***g*** for 15 min. and 100,000***g*** for 120 min.

Calcium phosphate columns (9 × 1·8 cm.) and buffers were prepared according to Taverne, Marshall & Fulton (1958). Scrapie mitochondrial fractions, intact and disintegrated by ultrasonic treatment, were prepared as for the DEAE-cellulose chromatography; and material derived from the equivalent of one mouse brain was applied to the columns which were eluted with 0·01 M-phosphate buffer (40 ml.) and then with successive 25 ml. portions of 0·1 M, 0·2 M, 0·4 M, and 1·0 M-phosphate buffer.

Determination of protein was carried out by the Lowry method (Lowry, Rosebrough, Farr & Randall, 1951).

RESULTS

Stability of the scrapie agent at different temperatures

The results of the experiment of heating the saline extracts of scrapie mouse brain are summarized in Fig. 1. A slightly lower titre was obtained in the samples heated for 10 and 60 min. at 62·5° than in those heated at 75°. A sharp decrease in the titre of the agent occurred when extracts were heated at 100°, but a few cases of scrapie were observed in mice inoculated with a 10^{-2} dilution of material heated at 100° even for 60 min., and one mouse in the group inoculated with material heated at 118° for 10 min. also contracted the disease.

The scrapie agent was also exceptionally stable when kept near −25°. Material held for 8 months in physiological saline, conditions that would be considered severe for most viruses, showed a decrease in ID 50 value from 6·7 to 5·5 only.

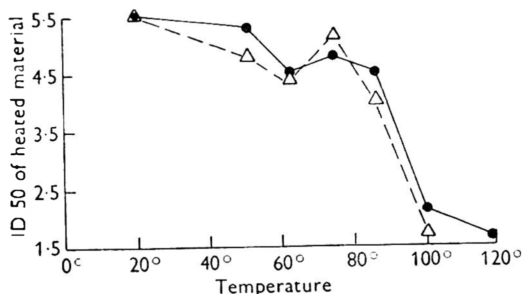


Fig. 1

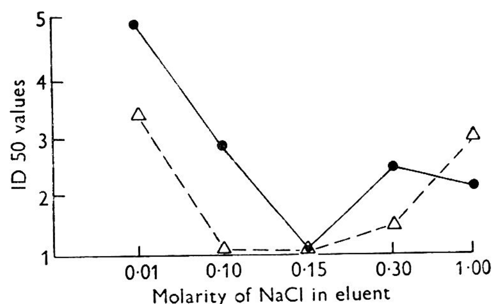


Fig. 2

Fig. 1. Effect of heat on saline extracts containing the scrapie agent. ID 50 values for the heated materials were determined by titration in mice as described in the text. ●—, extracts heated for 10 min.; △- - -, extracts heated for 60 min.

Fig. 2. Chromatographic behaviour on DEAE-cellulose of intact and ultrasonically-treated scrapie mouse brain mitochondrial fractions (SMF) ID 50 values for the various fractions were determined by titration as described in the text. Initial titre of the intact and ultrasonically-treated SMF = 4·8. △-- intact SMF; ●— ultrasonically treated SMF.

Effect of heat, ultrasonic treatment and Tween-80 on the sedimentation behaviour of the scrapie agent

As shown in Table 1, all these treatments had the remarkable property of making the agent more readily sedimentable than previously, and in the case of heat and Tween-80 treatment, the bulk of the agent became sedimentable on an ordinary bench centrifuge. The experiments were terminated after 5–6 months only (Hunter *et al.* 1963), so that the titres were relatively low. None of the treatments had much effect on the overall infectivity of the extracts, but in each case no activity at all was found in the non-sedimentable soluble fraction.

Chromatographic behaviour of the scrapie agent

When the intact mitochondrial fraction was chromatographed on DEAE-cellulose, only about 1 % of the infective material was recovered, although nearly a half of the added protein was eluted (Table 2). However (after ultrasonic treatment). as shown in Fig. 2, the fraction that passed straight through the column with the

Table 1. *Sedimentation of the scrapie agent from extracts treated in various ways*

Treatment	Initial titre (ID 50)	Titre in material successively sedimented at 500 *g*	10,000 *g*	100,000 *g*	ID 50 of final 'soluble' fraction
Heat (75–80°)	5·2	5·0	2·0	< 1·5	< 1·5
Ultrasonic	5·3	—	5·2	1·7	< 1·5
Tween-80 (0·01 %)	4·5	4·5	3·2	< 1·5	< 1·5

initially applied buffer had lost no infectivity. Recovery of applied protein was about 75 % in the case of the ultrasonically-treated material.

Very similar results (Table 3) were obtained when the scrapie agent was chromatographed on calcium phosphate. As in the case of the DEAE-cellulose run, much protein was retained when the intact mitochondrial fraction was used, and the small amount of scrapie agent that was eluted came in the fractions removed with the higher concentrations of phosphate buffer. The situation was altered in the

Table 2. *Recovery of protein from intact and ultrasonically-treated mitochondrial-lysosomal fractions after chromatography on DEAE-cellulose*

The total protein content of the material applied to the column was 24·3 mg. in each case.

Fraction eluted with	Intact fraction (mg. protein)	Ultrasonically-treated fraction (mg. protein)
0·01 M-phosphate buffer	3·0	13·0
0·1 M-NaCl	2·7	2·0
0·15 M-NaCl	1·5	1·0
0·3 M-NaCl	2·0	1·4
1·0 M-NaCl	1·5	0·0

Table 3. *Chromatography of intact and ultrasonically-treated scrapie brain mitochondrial-lysosomal fraction on calcium phosphate*

The intact mitochondrial fraction applied to the column had an ID 50 value of 5·9 when titrated in mice; the ID 50 value of the ultrasonically-treated material (a separate experiment calculated after 6 months only) was 4·7.

Fraction eluted with phosphate buffer of molarity	Titre (ID 50) from intact mitochondria	Titre (ID 50) from ultrasonically-treated mitochondria
0·01	< 1·7	3·7
0·1	< 1·5	3·7
0·2	2·4	2·5
0·4	3·5	< 2·7
1·0	3·3	< 2·5

case of the ultrasonically-treated fraction; again a substantial amount of the scrapie agent passed readily through the column. However, the overall recovery of infective agent was not as complete in the case of the calcium phosphate column as with DEAE-cellulose, even when ultrasonically-treated material was used.

Table 4. *Differential centrifugation of the fraction eluted from the DEAE-cellulose with 0·01 M-phosphate buffer*

Fraction	ID50 value	Protein content (mg.)
Ultrasonically-treated mitochondria applied to the column	5·5	—
Material eluted from the column with 0·01 M-phosphate buffer	4·7	—
Material in eluate sedimenting at 10,000 *g*.	4·1	2·4
Material in eluate sedimenting at 100,000 *g*.	4·2	7·7
Supernatant fluid above 100,000*g* sediment	3·2	2·9

In a further experiment where an ultrasonically-treated mitochondrial suspension from scrapie mouse brain was chromatographed on DEAE-cellulose, the material passing directly through the column in the 0·01 M-phosphate buffer was further fractionated by differential centrifugation (Table 4). The titres of the scrapie agent were now very similar in both 'mitochondrial' and 'microsomal' fractions. The column had, of course, removed some of the coarser debris, so that the 'microsomal' fraction sedimenting at 100,000*g* was bulkier in this case than the 'mitochondrial' fraction. There appeared to be a slight overall loss in infectivity in this experiment when the agent was passed through the DEAE-cellulose column.

DISCUSSION

In the heating experiment (Fig. 1) it seems probable that the relatively small losses in infective agent observed when the saline extracts were heated at 50° and 62·5° were occasioned by enzymic attack. At these temperatures, some degradative enzymes would be inactivated only slowly. On the other hand, at 75° most enzymes would be rapidly inactivated, although the even smaller losses of the scrapie agent observed at this temperature might also be due to the same cause. In fact, in repetitions of part of this experiment (see Table 1; also unpublished work), the loss of infective agent in extracts of scrapie heated to 75°–80° was negligible. It is interesting to note that substantial heat denaturation only sets in at temperatures near the melting temperature of DNA. However, it seems unlikely that the scrapie agent is a free nucleic acid since it has been found (Hunter & Millson, unpublished work; Mould *et al.* 1964; Pattison & Millson, 1960) that both RNase and DNase have only slight actions on the intact agent. When allowance is made for the probable action of enzymes, the heat denaturation curves are very smooth and the agent as a whole does exhibit remarkable resistance to the action of elevated temperatures. There does not seem to be any evidence here in favour of the existence of a heat-stable form of the agent, analogous to the bacterial spore; the results in fact suggest that the scrapie agent exists predominantly in a single form.

Previous experiments (Hunter *et al.* 1964) showed that when a cellular fractionation of mouse scrapie brain was done, the bulk of the infective agent was found in the mitochondrial-lysosomal fraction. Mould *et al.* (1964) pointed out that the smaller microsomal fractions contain almost as high a concentration of the scrapie

agent as does the mitochondrial-lysosomal fraction; they further suggest that 'the mouse scrapie agent, if particulate, is extremely small and closely bound to tissue components'. Our chromatographic work would lead towards the same view, although there does not seem to be any direct evidence at present to suggest that the agent is smaller than particles of the 'picornavirus' group. It is nevertheless difficult to explain the altered chromatographic behaviour of the scrapie agent after ultrasonic treatment on any basis other than the release of the agent in a particulate form, or bound to particulate debris, of a size much smaller than the mitochondria and lysosomes. Moreover, removal of the coarser debris during the passage of the agent through DEAE-cellulose (Table 4) certainly leads to the elution of the agent in a form less readily sedimentable than previously. It almost seems that the sedimentation of the agent is a property of the debris to which it is bound, rather than to any property of the agent itself.

The avid binding of the agent to a denatured debris after heat and Tween treatment is remarkable, and makes the task of concentrating and separating the agent difficult. It again suggests that the agent is small, but it is still possible that the agent is, for instance, a small arbovirus with unusual surface properties. It is of interest here that the scrapie agent is sensitive to a considerable degree to the action of ether (Eklund, Hadlow & Kennedy, 1963; Hunter & Millson, 1964), and also to the action of other organic solvents (Hunter & Millson, unpublished work), and it seems possible that lipid materials are concerned in maintaining the surface structure of the agent.

The results obtained when using calcium phosphate columns lead to the same general conclusions about the nature of the scrapie agent. Ultrasonic treatment seems to be equivalent to ether treatment (Mould, Smith & Dawson, 1964), although ether treatment involves a greater loss in infectivity.

The authors are grateful to Dr W. S. Gordon, C.B.E., for his sustained interest and advice, to Mr A. Mackenzie and Mr P. Dennis for preparing the histological specimens, and they acknowledge the skilled technical assistance of Miss S. Langford and Mr M. Vockins. The work was supported in part by grants made to the Agricultural Research Council from U.S. Government funds administered under U.S. Public Law 480.

REFERENCES

CHANDLER, R. L. (1963). Experimental scrapie in the mouse. *Res. vet. Sci.* **4**, 276.

EKLUND, C. M., HADLOW, W. J. & KENNEDY, R. C. (1963). Some properties of the scrapie agent and its behaviour in mice. *Proc. Soc. exp. Biol., N.Y.* **112**, 974.

HUNTER, G. D. & MILLSON, G. C. (1964). Further experiments on the comparative potency of tissue extracts from mice infected with scrapie. *Res. vet. Sci.* **5**, 149.

HUNTER, G. D., MILLSON, G. C. & CHANDLER, R. L. (1963). Observations on the comparative infectivity of cellular fractions derived from homogenates of mouse-scrapie brain. *Res. vet. Sci.* **4**, 543.

HUNTER, G. D., MILLSON, G. C. & MEEK, G. (1964). The intracellular location of the agent of mouse scrapie. *J. gen. Microbiol.* **34**, 319.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. J. (1951). Protein measurement with the Folin phenol reagent. *J. biol. Chem.* **193**, 265.

MOULD, D. L., DAWSON, A. McL. & SMITH, W. (1964). The infectivities of brain-cell fractions from mice infected with Suffolk sheep-scrapie agent. *Biochem. J.* **91**, 13P.

MOULD, D. L., SMITH, W. & DAWSON, A. McL. (1964). The elution of scrapie brain tissue through calcium phosphate columns. *Biochem. J.* **91**, 13P.

PARKER, R. C. (1959). *Methods of Tissue Culture* (3rd edition), p. 253. London: Pitman Medical Publishing Co. Ltd.

PATTISON, I. H. & MILLSON, G. C. (1960). Further observations on the experimental production of scrapie in goats and sheep. *J. comp. Path.* **70**, 185.

PATTISON, I. H. & SMITH, K. (1963). Histological observations on experimental scrapie in the mouse. *Res. vet. Sci.* **4**, 269.

SCHNEIDER, H. A. (1959). Genetic and nutritional aspects of experimental 'allergic' encephalomyelitis, in *'Allergic' Encephalomyelitis*, ch. 3, p. 130. Ed. by M. W. Kies and E. C. Alvard. Oxford: Blackwell Scientific Publications, Ltd.

STAMP, J. T. (1962). Scrapie; a transmissible disease of sheep. *Vet. Rec.* **74**, 357.

TAVERNE, J., MARSHALL, J. H. & FULTON, F. (1958). The purification and concentration of viruses and virus soluble antigens on calcium phosphate. *J. gen. Microbiol.* **19**, 451.

J. gen. Microbiol. (1964), 37, 259–266
Printed in Great Britain

Synchrony and the Elimination of Chance Delays in the Growth of Poliovirus

By P. D. COOPER*
Virus Culture Laboratory, Medical Research Council Laboratories, Carshalton, Surrey

(*Received* 11 *June* 1964)

SUMMARY

The effect of multiplicity of infection on the growth kinetics of poliovirus suggests that the eclipse period usual for singly infected cells (3·6–3·8 hr) includes variable delays averaging about 1 hr. These delays are overcome at multiplicities above 3, and are sometimes spontaneously absent. They are ascribed largely to chance effects, and lead to markedly asynchronous maturation of virus; in their absence, maturation is almost synchronous.

The kinetics of acid-irreversible eclipse and of the development of antiserum resistance show that about half of the delays must occur during viral penetration; in support of this, virus growth initiated with infective RNA is 0·5 hr less delayed than that of intact virus, although otherwise similar. However, infective RNA synthesis was not detected earlier than 2 hr after infection, even in the absence of chance delays.

INTRODUCTION

The events to be observed during a single cycle of virus growth usually form a reproducible sequence. However, even if the cells are infected simultaneously, all cells may not reach a given part of the sequence at the same time (Cairns, 1957). Such asynchrony occurs in many viral systems, and may at times hinder the study of virus growth as much as non-simultaneous infection of the cells. One cause of asynchrony is the presence of chance hesitations between growth steps ('random or variable delays') and it is important in studying virus growth to know the average duration of the viral processes as compared with such delays. It is theoretically possible, for example, that the eclipse period results solely from very large random delays.

This work investigates the extent of delays and asynchrony in the growth of poliovirus in ERK cells, and ways of avoiding them. Of the eclipse period of 3·6 hr which is usual at single multiplicity of infection, nearly 1 hr may be regarded as occupied by random delays. These delays result in substantial asynchrony of virus maturation, and a considerable portion of them appears to occur during stages of penetration. However, random delays and asynchrony are sometimes absent, probably because of factors affecting the state of the cells, which are not understood. Random delays are also eliminated by large multiplicities of infection, which result in considerable synchrony of viral maturation and a reproducibly short eclipse period of 2·6–2·7 hr.

* Present address: Department of Microbiology, John Curtin School of Medical Research, Australian National University, Canberra, Australia.

METHODS

Virus. Poliovirus type 1 (strain Brunenders) was used; virus and infective cells were assayed by the agar cell-suspension plaque method (Cooper, 1961). Infective RNA assays used the procedure described by Cooper (1962). All host cells were from the ERK line grown in medium CSV6 (Cooper, Wilson & Burt, 1959).

One-step growth method. ERK cells were resuspended from bottle cultures with trypsin (2·5 mg./ml.) plus EDTA (2 mg./ml.) to give a monodisperse suspension, and maintained overnight as a suspension culture at 36°. Cells were infected to various multiplicities in suspension at 0°, washed and added to medium at 37°. The pH was controlled at pH 7·3 by replacement of glucose with galactose and by gassing with 5 % CO_2 in air. The cells were kept in suspension by a 'New Brunswick' type of rotary shaker. The final cell suspension was assayed for total cells ($5-10 \times 10^5$/ml.), infective cells (50–80 % of total cells when fully infected) and virus free (less than 5 % of infective cells); < 10 % of the cells were in clumps of 2 or more, and < 5 % were non-viable. The multiplicity of infection was determined from assays of the virus added and of the virus recovered after washing the cells. Samples were taken at intervals during virus growth into 2 mg./ml. deoxycholate for assay of cell-associated mature virus. Growth is expressed in terms of plaque-forming units (p.f.u.) produced per infective cell, and the eclipse period equals the time from addition to warm medium to the time of maturation of 1 p.f.u. per infective cell.

Sampling procedure for determination of yield per yielding cell. Portions of infected cell suspensions were taken during 1-step growth, chilled and diluted to 5, 10 and 20 infective cells/ml.; 0·05 ml. samples of these dilutions were added to each cup of an 80-cup haemagglutinin tray, and then frozen in solid CO_2 and thawed. Fresh cells were added to each cup (0·05 ml. containing 10^6 cells), followed by 0·1 ml. of molten agar medium. The cup contents were very rapidly mixed, removed and dropped on to chilled agar base layers contained in 4 in. Petri dishes; the drops were spread somewhat before they set. Eight samples were accommodated per dish, and 600–700 samples were plated in 4–5 hr on 80 Petri dishes. The plates were then incubated as for a normal plaque assay, when the plaques developing in each sample could be counted; only those dilutions in which about 50 % of the samples contained plaques were selected for counting.

RESULTS

The effect of multiplicity of infection on the growth of poliovirus. If there are variable delays in a viral growth process caused purely by probability effects, then increasing the multiplicity of infection should decrease the overall chance of delay for any given infected cell (Cairns, 1957). The eclipse period at single multiplicity was delayed to 3·6 hr but increasing the number of adsorbed particles decreased the eclipse period to 2·6–2·7 hr (Fig. 1). These values were quite reproducible, and eclipse periods were never less than 2·6 hr however high the multiplicity (maximum tested equals 30). Figure 2 suggests that the eclipse periods are made up of a component of up to 1 hr which is variable with multiplicity plus an invariable component of 2·7 hr. The shortening of the eclipse period was negligible above a multiplicity of 3. An arithmetical plot (Fig. 3) of the data of Fig. 1 shows that the rate of maturation tends to be constant and that, at very low multiplicities, virus matures

more slowly. Similar curves were found for influenza virus under conditions of delay and asynchrony (Cairns, 1957). Darnell (1958) and Howes (1959, *a*, *b*) reported that higher multiplicities increased the growth rate of poliovirus.

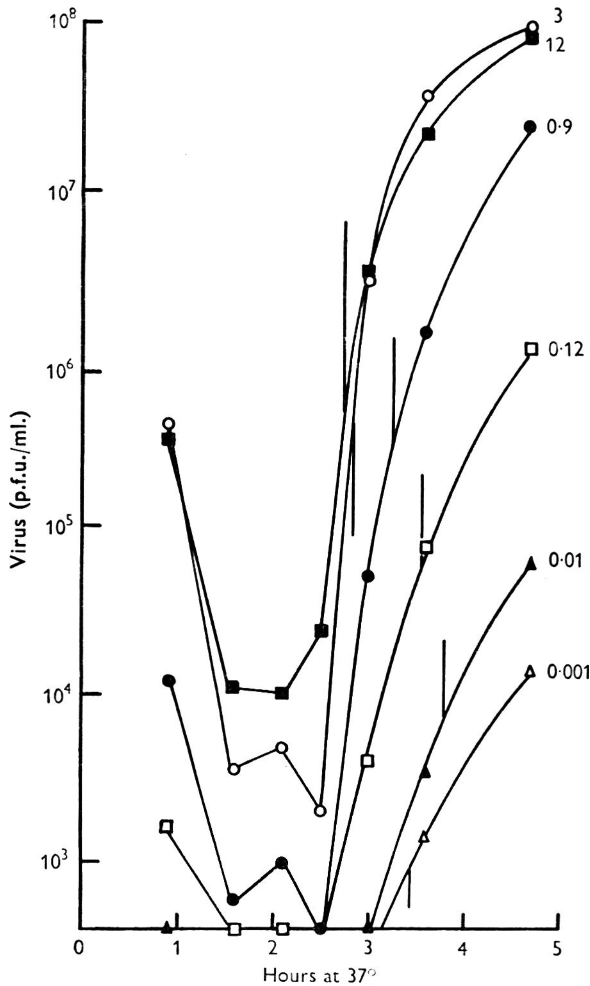


Fig. 1. One-step 'delayed state' maturation curves of poliovirus in suspended ERK cells at 37°. Six cultures from one batch of cells were simultaneously infected with various multiplicities of virus, which are indicated on the figure. The short vertical lines join each curve at the point where the internally matured virus equals the infective cell count (end of eclipse period).

However, in rather less than half of our experiments the growth rate at single multiplicity of infection did not show any delay (Fig. 4): the eclipse period was 2·7 hr, maturation was rapid and exponential in rate, and increasing the multiplicity had no effect. The reasons for this lack of delay are not known, but are suspected to lie in the metabolic state of the cell. In the growth curves of Fig. 4, the increase of infective RNA was also measured for the two cultures, and was the same for both. The RNA curve from the lower multiplicity culture is shown, and indicates

that, even in the absence of delay, an increase in infective RNA was not detected earlier than 2 hr after infection. This aspect is dealt with more fully in the accompanying paper (Cooper, 1964).

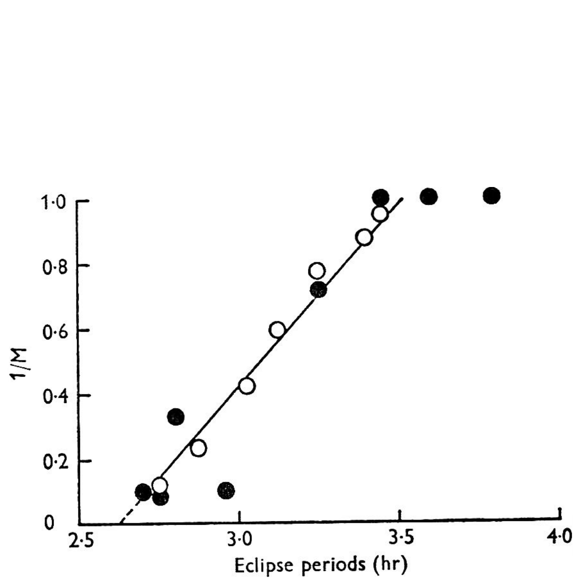


Fig. 2

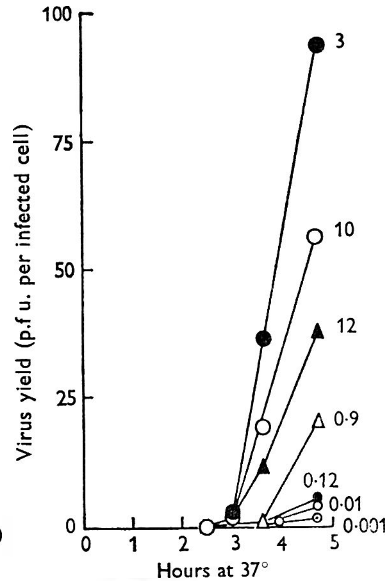


Fig. 3

Fig. 2. The relation for poliovirus between the reciprocal of the multiplicity of infection (1/M) and the eclipse period. One experiment (●) is that of Fig. 1; the other (○) is a replicate. Multiplicity of infection in this case equals the average number of particles adsorbed per *infected* cell (minimum equals 1), and is calculated assuming a Poisson distribution of virus among cells.

Fig. 3. One-step 'delayed state' maturation curves of poliovirus in suspended ERK cells at 37°. The data are the same as those of Fig. 1, and the multiplicities of infection are indicated on the figure.

Synchrony and asynchrony in maturation of poliovirus. The synchrony of maturation was examined by the following direct method, using delayed cultures and cultures in which delay was spontaneously absent or was eliminated by a high multiplicity of infection. One-step growth experiments were performed in which samples of intact cells were taken at times covering the estimated end of eclipse (2·5–4 hr). A series of dilutions of each of these samples was made, and 0·05 ml. of each dilution was added to every cup of an 80-cup haemagglutinin tray. The dilutions were such that, for each sample, at least 1 tray contained an average of about 1 infective cell per two cups. After freezing the samples in the trays and thawing to liberate the virus, the contents of each cup were plated by the procedure already described. The assays from those trays in which about half the cups yielded no or negligible virus enabled the infective virus content of individual cells to be calculated. Three representative experiments are shown in Table 1. In Exp. 1 the eclipse period at single multiplicity of infection was 2·6 hr and virus maturation was rapid and almost exponential, and so random delays were presumed to be absent. The degree of asynchrony was in fact very small; the distribution of virus

among cups was approximately Poissonian, and the number of cups yielding virus at the end of the eclipse period was almost that to be expected from the infective cell assay, so that almost all potentially yielding cells contained some virus at this time.

Table 1. *The distribution of poliovirus among cells of three cultures experiencing different random delay effects*

The one-step growth and sampling procedures are described in the Methods.

Experiment no.			1		2	3
Multiplicity of infection			0·01		0·01	5
Eclipse period (hr)			2·6		3·7	2·6[a]
Presumed state			Non-delayed		Delayed	Delay overcome by high multiplicity
Time of sampling (hr)		2·75	3·0	5·0	4·0	3·0
Yield per yielding cell						
(*a*) From average of positive samples		1·25	1·4	100–200	3·0	6·0
(*b*) Expected from growth curve		1·1	3·3	150	2·5	6·5
Plaques per sample (each sample is presumed to contain only 1 infective cell)	p.f.u.	No. of samples				
	0	53	44	0	102	87
	1–3	27	34 }	27†	12	18
	4–10	0	0 }		9	42
	11–20	0	0 }		9	13
	21–30	0	0	0	4	0
	31–40	0	0	0	3	0
	41–50	0	0	0	1	0
	51–70	0	0	5	0	0
	71–80	0	0	0	2	0
	80–100	0	0	0	0	0
	> 100	0	0	24	0	0

[a] A replicate experiment performed simultaneously but at single multiplicity had an eclipse period of 3·6 hr.
† Released virus.

In Exp. 2, the maturation rate was slow and the eclipse period was 3·7 hr; random delays were presumed to be present. This experiment showed much more asynchrony than the first in that the yields per cell varied widely and the proportion of cells yielding no virus was higher than that to be expected from the average yield per cup. In Exp. 3, the culture examined had a multiplicity of 5, and an eclipse period of 2·6 hr, while a replicate culture at single multiplicity grown simultaneously had an eclipse period of 3·6 hr. Hence it is presumed that these cultures were in a state in which each adsorbing particle would be subject to random delays but that such delays were overcome for the infective cell by a high multiplicity. Table 1 shows that overcoming the delays in this way also removed the asynchrony. Thus delayed cultures were highly asynchronous in maturing virus, while cultures with little intrinsic delay or in which delay was overcome by high multiplicity were much less asynchronous.

Delays in entering acid-irreversible eclipse. Of several stages detected during the penetration of poliovirus, the transition to non-recoverability of infectivity with pH 2·5 buffer ('acid-irreversible eclipse') appears to be the last (Fenwick & Cooper, 1962). The rate of acid-irreversible eclipse in the system used (Fig. 5) shows that the time taken to inactivate the virus in this way can occupy a significant portion of the eclipse period, as 50 % of the particles in Exp. A were delayed by at least 0·5 hr. However, the rate of eclipse was not reproducible between experiments (in Fig. 5, curve B, the 50 % time was only 0·15 hr) indicating that delays involved in eclipse were variable and were less in some experiments than in others.

Delays in development of antiserum resistance. To see whether delays were introduced at a stage earlier than acid-irreversible eclipse, the rates of development of antiserum resistance of the infective cell were compared at different multiplicities of infection. Multiplicity had a marked effect and the development of antiserum

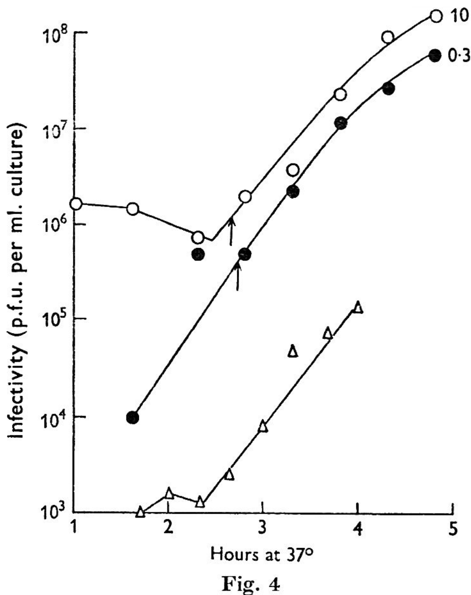


Fig. 4

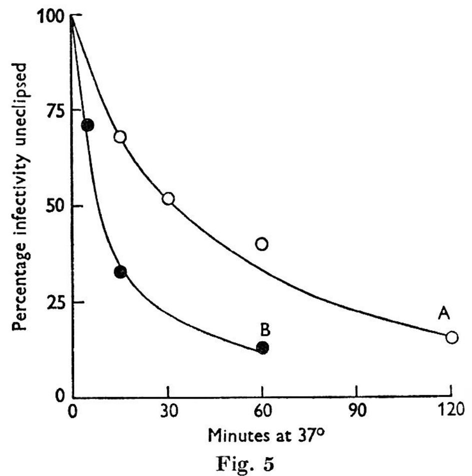


Fig. 5

Fig. 4. One-step 'non-delayed state' growth curves of poliovirus in suspended ERK cells at 37°, showing increase in total mature virus from multiplicity of infection of 10 (○) and 0·3 (●), and increase of infective RNA from multiplicity of 0·3 (△). The arrows indicate the end of the eclipse periods.

Fig. 5. The rate of acid-irreversible eclipse of poliovirus in two replicate experiments (A and B), using suspended ERK cells at 37°. Infected cells were diluted at intervals through 0·05 M-glycine (pH 2·5) for assay of uneclipsed infectivity.

resistance was faster at a high multiplicity (Fig. 6). With 10 p.f.u./cell, the transition of almost all cells occurred within a few minutes. As the maximum hastening of antiserum resistance by high multiplicity was only 20–30 min., this source of delay possibly accounts for rather less than half of the delays usually present. However, in several single-multiplicity growth experiments all infective cells became resistant to antiserum in 1–5 min., indicating that the delays involved in this growth step are also variable between, as well as within, experiments.

Comparison of growth curves with virus and with infective RNA as inocula. If the uncoating of the virion leads to variable delays in the growth cycle, then infection of the cell with uncoated genetic material (infective RNA) should reduce this delay. The eclipse period and the time taken to *release* 1 p.f.u./cell were both 0·5–0·8 hr shorter when using infective RNA than when using intact virus at single multiplicity (Fig. 7). Hence a considerable portion of the delay occurs during the uncoating of the virus.

A control experiment was necessary for the interpretation of Fig. 7. This was because independent experiments with infective RNA labelled with ^{32}P showed that as much as 5 % of the RNA was taken up by the cells when the RNA was added at

high concentration. Thus it was possible that the virus deriving from infective RNA in Fig. 7 did not contain newly synthesized RNA, but inoculum RNA which had been 'recoated'. This possibility was disproved by preparing two replicate batches of virus labelled with ^{32}P, using non-radioactive infective RNA (extracted from virus at a concentration of 10^{10} (p.f.u./ml.) and non-radioactive intact virus respectively as inocula. The procedure for virus growth and purification described by Fenwick & Cooper (1962) was used; the viral radioactivity was isolated from both preparations by means of a potassium tartrate density gradient, and their specific activities were found to be almost identical.

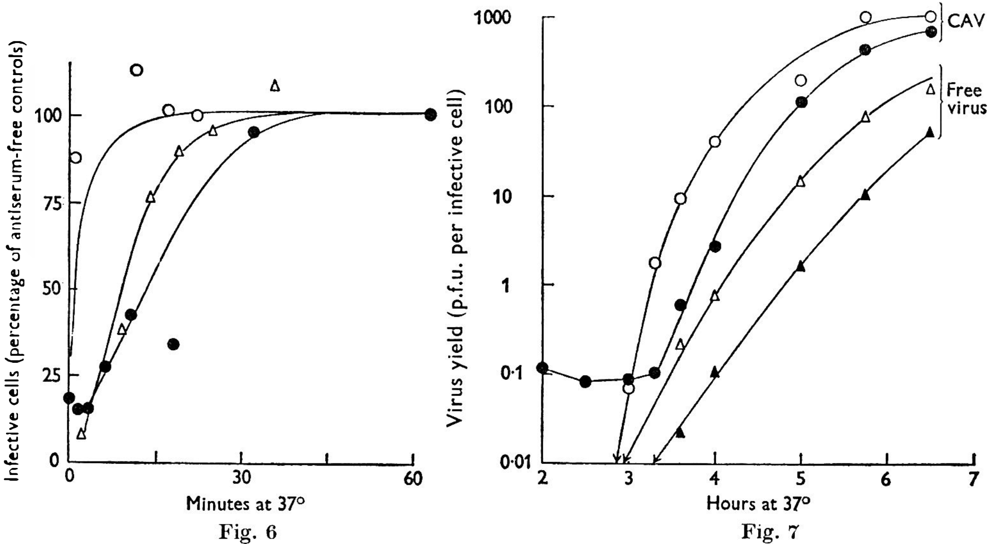


Fig. 6. Effect of multiplicity of infection of poliovirus on the development of antiserum resistance of suspended ERK cells at 37°. Multiplicities were 10 (○), 2·5 (△), and 0·01 (●). Infected cells were diluted at intervals into sufficient antiserum to neutralize 99 % of free virus in 10 min. at 4°. After 30 min., the cells were diluted free of antiserum and plated as for virus. The cells surviving as infective centres are expressed as a percentage of cell samples containing no antiserum.

Fig. 7. One-step growth curves of poliovirus initiated by infective RNA (○ = cell-associated virus, or CAV; △ = released virus) and by intact virus (● = CAV; ▲ = released virus). Both sets of cultures had been treated identically before infection, including washing with 0·8 M-Na_2SO_4 at pH 8·0; they were infected as monolayers at 15°, washed rapidly several times with PBS at 37° in a room at 37°, resuspended with trypsin and versene mixture, and washed before adding to warm growth medium. Time zero equals the time of transition to 37°; care was taken that this transition was sharp and permanent.

DISCUSSION

These data indicate that the normal single multiplicity eclipse period of poliovirus, 3·6–3·8 hr in the system studied, includes variable delays of about 1 hr. At least half but not all of these delays occur during penetration of the virus, probably after the reversible 'neutralization' of virus by cells which occurs with little delay (Fenwick & Cooper, 1962). The term 'penetration' is used here to include all stages up to the final uncoating of the genetic material. The delays lead to considerable asynchrony of maturation. Howes (1959*a*, *b*) estimated that there was a total spread of

2–3 hr for the termination times of individual eclipse periods of poliovirus in his system. However, delays and concomitant asynchrony are sometimes spontaneously absent, or can be overcome by a high multiplicity of infection; criteria of such effects are a short eclipse period and rapid maturation. Variations in the extent of delay may explain the differences in kinetics of poliovirus growth apparent between other reports (Dulbecco & Vogt, 1955; Darnell, 1958; Howes, 1959*a*, *b*; Holland, Hoyer, McLaren & Syverton, 1960; Darnell, Levintow, Thorén & Hooper, 1961). It is clearly desirable, particularly in biochemical studies of virus growth, to eliminate asynchrony during the infective process, and this now appears to be feasible by use of the method described above. An implication of these findings is that practically all of the 2·7 hr minimum eclipse period of poliovirus must be occupied by viral growth processes, rather than by chance hesitations between processes.

It should be noted that the eclipse periods would also be shortened by high multiplicities if each adsorbing particle were able to replicate autonomously but without delays (Cooper, 1958). In this case the variety of individual multiplicities of infection in a culture would yield considerable asynchrony, again resembling the effects of random delay. However, the acid-irreversible eclipse curves and growth curves resulting from infective RNA indicate that much delay *must* be present during penetration, and hence that the contribution of autonomous growth to the effects found with poliovirus must be correspondingly limited. The fact that increasing the multiplicity above 3 does not further shorten the eclipse period also indicates that fully autonomous growth is not permitted.

The excellent assistance of Mr H. Cumming and Miss J. Constable is gratefully acknowledged.

REFERENCES

CAIRNS, H. J. F. (1957). The asynchrony of infection by influenza virus. *Virology*, **3**, 1.

COOPER, P. D. (1958). 'Shortened latency' as a result of multiple infection by vesicular stomatitis virus in chick cell culture. *J. gen. Microbiol.* **19**, 340.

COOPER, P. D. (1961). An improved agar cell-suspension plaque assay for poliovirus: some factors affecting efficiency of plating. *Virology*, **13**, 153.

COOPER, P. D. (1962). Studies on the structure and function of the poliovirion: effects of concentrated urea solutions. *Virology*, **16**, 485.

COOPER, P. D. (1964). The kinetics of the appearance of poliovirus ribonucleic acid. *J. gen. Microbiol*, **37**, 2.

COOPER, P. D., WILSON, J. N. & BURT, A. M. (1959). The bulk growth of animal cells in continuous suspension culture. *J. gen. Microbiol.* **21**, 702.

DARNELL, J. E. JR. (1958). Adsorption and maturation of poliovirus in singly and multiply infected HeLa cells. *J. exp. Med.* **107**, 633.

DARNELL, J. E. JR., LEVINTOW, L., THORÉN, M. M. & HOOPER, J. L. (1961). The time course of synthesis of poliovirus RNA. *Virology*, **13**, 271.

DULBECCO, R. & VOGT, M. (1955). Biological properties of poliomyelitis viruses as studied by the plaque technique. *Ann. N.Y. Acad. Sci.* **61**, 790.

FENWICK, M. L. & COOPER, P. D. (1962). Early interactions between poliovirus and ERK cells: some observations on the nature and significance of the rejected particles. *Virology*, **18**, 212.

HOLLAND, J. J., HOYER, B. H., MCLAREN, L. C. & SYVERTON, J. T. (1960). Enteroviral ribonucleic acid. I. Recovery from virus and assimilation by cells. *J. exp. Med.* **112**, 821.

HOWES, D. W. (1959*a*). The growth cycle of poliovirus in cultured cells. II. Maturation and release of virus in suspended cell populations. *Virology*, **9**, 96.

HOWES, D. W. (1959*b*). The growth cycle of poliovirus in cultured cells. III. The asynchronous response of HeLa cells multiply infected with type I poliovirus. *Virology*, **9**, 110.

J. gen. Microbiol. (1964), **37**, 267–270
Printed in Great Britain

The Kinetics of the Appearance of Poliovirus Ribonucleic Acid

By P. D. COOPER*

Virus Culture Laboratory, Medical Research Council Laboratories, Carshalton, Surrey

(*Received* 11 *June* 1964)

SUMMARY

One-step growth conditions were used in which cells began replication of poliovirus RNA almost synchronously, and in which factors limiting the rate of replication appeared to be absent. RNA of uneclipsed virus was destroyed by using light-sensitive inocula. Under these conditions, ribonuclease-sensitive infective RNA was detectable in unchanged amount for the first 2 hr of infection, at which time replication began abruptly; the increase of poliovirus RNA was geometrical for a further 1–2 hr.

INTRODUCTION

Where the kinetics can be determined, the rates of increase of several viral nucleic acids (measured chemically or by infectivity) appear in one-step growth experiments to be more or less constant with time. However, in phage T_2 (Hershey, Dixon & Chase, 1953) a genetic experiment (Luria, 1951) showed that DNA replication depends on a geometric mechanism. Rate-limiting factors may, therefore, have transformed a presumptively exponential rate of increase into an apparently linear one. Another complication having the opposite effect is that individual cells may not begin nucleic acid replication synchronously. In this case, an exponential rate of increase in the proportion of cells which begin replication could transform a presumptively linear rate of increase of nucleic acid into one which was apparently exponential in the initial phase.

This paper reports experiments on the rate of increase of poliovirus RNA under conditions lacking these complications. Random delays were largely absent so that maturation began almost synchronously (Cooper, 1964). Cells were infected with a light-sensitive inoculum (Wilson & Cooper, 1962). Illumination after a short 'pulse' of penetration gave additional synchrony and destroyed the RNA of virus uneclipsed during this period (Wilson & Cooper, to be published): the very early kinetics could be examined without their being obscured by excess of non-productive RNA. The appearance of new infective RNA began sharply at 2 hr; the increase was exponential for the next 2 hr, so that rate limiting factors were also absent.

* Present address: Department of Microbiology, John Curtin School of Medical Research, Australian National University, Canberra, Australia.

METHODS

Virus. Poliovirus type 1 (strain Brunenders) was used, grown and assayed in ERK cells (Cooper, Wilson & Burt, 1959; Cooper, 1961). Infective RNA was extracted and assayed by the method described by Cooper (1962); all RNA assays used two or three 3-fold dilutions, 4 in. Petri dishes and four replicates per dilution.

One-step growth method. The method used was described by Cooper (1964); monodisperse cells were infected to an adsorbed multiplicity of 10 at 0°, washed and added to medium at 37° at zero time. Serum was omitted from the medium. The cells were maintained in suspension by a rotary shaker. Inoculum virus was grown in neutral red, 4 μg./ml. and all operations including addition to warm medium were performed under a red light (Wilson & Cooper, 1962, 1963); after 5 min. at 37° the cultures were strongly illuminated for 10 min. with white light (proportion of virus surviving under these conditions equals 10^{-3}). Samples were taken for infective cell and free virus assay, and at intervals into 2 mg. deoxycholate/ml. for cell associated mature virus assay (no free virus was present, and no cell associated virus was found up to 2·3 hr after infection), and into aqueous phenol for extraction of RNA.

For all cultures the eclipse periods (time after infection of appearance of 1 intracellular p.f.u. per infective cell) were 2·6–2·7 hr and maturation was rapid and exponential. These criteria were regarded as essential to indicate the absence of random delays and asynchrony (Cooper, 1964).

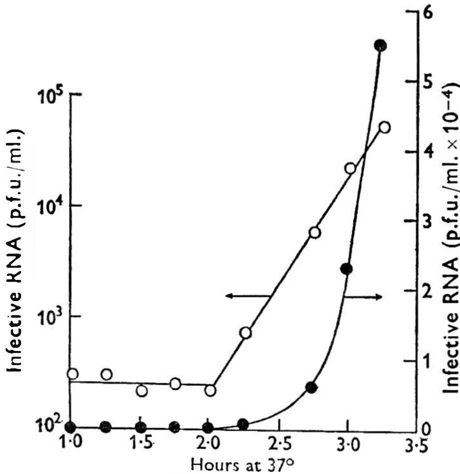


Fig. 1

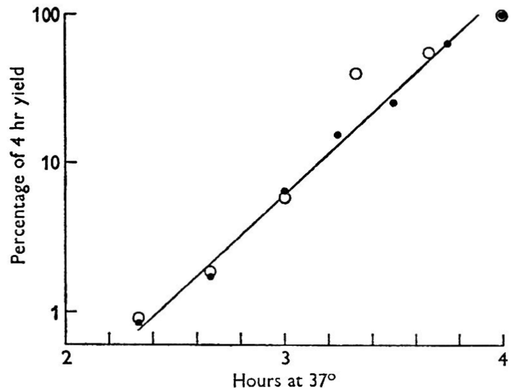


Fig. 2

Fig. 1. One-step growth curve of poliovirus infective RNA, plotted semilogarithmically (○, left-hand ordinate) and arithmetically (●, right-hand ordinate). Uneclipsed inoculum virus was destroyed by use of light sensitive inocula, and virus maturation was approximately synchronous.

Fig. 2. Two replicate one-step growth curves of poliovirus infective RNA, expressed as a percentage of the 4-hr yield. Conditions as for Fig. 1.

RESULTS

An intensive examination was made of the rate of increase of poliovirus RNA between 1 and 3 hr after infection (Fig. 1). The eclipse period, as determined by assays of mature virus and infective centres (not shown), was 2·6 hr. This indicates that maturation was approximately synchronous (Cooper, 1964), and hence that all cells must have begun replication of viral RNA well before this time. More than 60% of the RNA found up to 2 hr was sensitive to ribonuclease when the cells were disrupted and treated with the enzyme before phenolic extraction. Infection with virus grown in 4 μg. neutral red/ml. followed by white light has destroyed 99·9% of the RNA of uneclipsed virus (Wilson & Cooper, to be published). Therefore this initial RNA represents input nucleic acid which was uncoated but not degraded or eclipsed. RNA synthesis began sharply 2 hr after infection and increased exponentially up to 4·0 hr (Fig. 2). Increases in infective RNA generally continued up to 5 hr in this system; the increase in infective RNA was 10^3 to 10^4 times the initial (2 hr) value, whereas the yield of virus rarely exceeded 300 p.f.u./cell. It is presumed that this excess of infective RNA reflects the RNA built into particles which do not register as plaque-forming units, together with any RNA which is not matured.

DISCUSSION

These data indicate that poliovirus RNA can be shown to increase geometrically, provided that adequate technical safeguards are taken. This may reflect a truly geometric mechanism, in which each progeny RNA molecule is able to act as a template for new molecules. This conclusion is supported by the observations that the proportion of poliovirus recombinants increases during the growth cycle (Ledinko, 1963), and that more than one molecule per cell of the RNA of the closely similar EMC virus can exist in double-stranded form (Montagnier & Sanders, 1963). The underlying mechanism, however, may be no more than pseudogeometric. For example, each progeny RNA molecule may induce the synthesis of some substance which increases the rate of replication, conceivably viral RNA polymerase.

I am grateful to Mr H. Cumming and Miss J. Constable for skilful technical assistance.

REFERENCES

COOPER, P. D. (1961). An improved agar cell-suspension plaque assay for poliovirus: some factors affecting efficiency of plating. *Virology*, **13**, 153.

COOPER, P. D. (1962). Studies on the structure and function of the poliovirion: effect of concentrated urea solutions. *Virology*, **16**, 485.

COOPER, P. D. (1964). Synchrony and the elimination of chance delays in the growth of poliovirus. *J. gen. Microbiol.* **37**, 2.

COOPER, P. D., WILSON, J. N. & BURT, A. M. (1959). The bulk growth of animal cells in continuous suspension culture. *J. gen. Microbiol.* **21**, 702.

HERSHEY, A. D., DIXON, J. & CHASE, M. (1953). Nucleic acid economy in bacteria infected with bacteriophage T2. I. Purine and pyrimidine composition. *J. gen. Physiol.* **36**, 777.

LEDINKO, N. (1963). Genetic recombination with poliovirus type 1. Studies of crosses between a normal horse serum-resistant mutant and several guanidine resistant mutants of the same strain. *Virology*, **20**, 107.

Luria, S. E. (1951). The frequency distribution of spontaneous bacteriophage mutants as evidence for the exponential rate of phage reproduction. *Cold Spr. Harb. Symp. quant. Biol.* **16**, 463.

Montagnier, L. & Sanders, F. K. (1963). Replicative form of encephalomyocarditis virus ribonucleic acid. *Nature, Lond.* **199**, 664.

Wilson, J. N. & Cooper, P. D. (1962). Photodynamic demonstration of two stages in the growth of poliovirus. *Virology*, **17**, 195.

Wilson, J. N. & Cooper, P. D. (1963). Aspects of the growth of poliovirus as revealed by the photodynamic effects of neutral red and acridine orange. *Virclogy*, **21**, 135.

J. gen. Microbiol. (1964), 37, 271–275
Printed in Great Britain

Toxicity of the Agents of Trachoma and Inclusion Conjunctivitis

By JANICE TAVERNE, W. A. BLYTH AND P. REEVE
M.R.C. Trachoma Research Unit, Lister Institute of Preventive Medicine, London, S.W. 1

(*Received* 3 *June* 1964)

SUMMARY

The toxicities of strains of trachoma and inclusion conjunctivitis viruses differing in virulence for the chick embryo were compared at different times during their growth in the chick embryo yolk sac. As measured by the ability to kill mice and to induce skin lesions in guinea-pigs, toxicity increased until the time at which embryos began to die. All strains possessed similar particle:toxin ratios. It is considered unlikely that the differing virulence of the strains depends on differences in the amount of toxin per elementary body.

INTRODUCTION

Reeve & Taverne (1963) found that for a given dose some strains of trachoma and inclusion conjunctivitis (TRIC) agents kill chick embryos more quickly than others. One explanation suggested was that elementary bodies of fast-killing strains possess more toxin. To test this we compared the toxicity of three fast-killing and three slow-killing TRIC agents. Since comparisons between strains are not valid unless made when toxin to particle ratios are maximum, measurements were made throughout the growth of each strain in the chick embryo yolk sac. Toxicity was titrated by the ability to kill mice injected intravenously and to form skin lesions in guinea-pigs injected intracutaneously; titres were compared with the total number of elementary bodies present in the suspension.

METHODS

TRIC agents are named according to the system proposed by Gear, Gordon, Jones & Bell (1963). Their original names used in this paper are given in brackets. Fast-killing variants are suffixed *f*.

TRIC/China/Peking-2/OT *f* (T'ang, Chung, Huang & Wang, 1957).

TRIC/2/SAU/HAR-2/OT (SA 2) (Murray *et al.* 1960) received from Miss L. Hanna, University of California Medical Center, San Francisco.

The variant SA 2 *f* derives from material received from Dr S. Bell of the School of Public Health, Harvard University.

TRIC/ J J /WAG/MRC-1/OT (G 1) (Collier & Sowa, 1958).

TRIC/ J J /GB/MRC-4/ON (LB 4) (Jones, 1961) and the variant LB 4 *f*.

Virus culture. Strains were grown in yolk sacs of 7-day chick embryos kept at 35° and candled daily.

Diluent. Phosphate buffered saline (Dulbecco & Vogt, 1952) containing streptomycin sulphate 1000 μg./ml. was used.

Virus suspensions. 10 or 20 % (w/v) suspensions were made by shaking infected yolk sacs in diluent and then discarding the membranes.

Particle counts were made by the dark field method of Reeve & Taverne (1962), except that treatment with trypsin was omitted, and suspensions were purified by centrifuging at 8000*g* for 20 min. in M-KCl.

Mouse test. Two-fold dilutions of virus suspensions were injected in 0·5 ml. volumes into the tail veins of T.O. mice weighing 10–15 g. Deaths occurring immediately were regarded as non-specific; those occurring up to 48 hr were used to measure the 50 % lethal dose (mouse LD50) calculated by the method of Reed & Muench (1938).

Guinea-pig test. 'Half-log' (1/3·16) dilutions of virus suspensions were made. The backs of adult white guinea-pigs weighing 300–400 g. were depilated and 0·2 ml. volumes of suspension injected intracutaneously. Virus suspensions induced erythematous indurations that reached maximum intensity at 24 hr. With high concentrations, lesions became necrotic.

The diameter of erythema varied directly with dilution of virus suspension. With a given suspension there was little variation between the diameters of lesions induced on the backs of ten guinea-pigs or between lesions induced at different places on the backs of individual animals. The standard error was less than 10 % whether a lesion of 10 or 5 mm. diameter was taken as the end-point. For greater sensitivity the end-point chosen was the reciprocal of the highest dilution giving a lesion with a diameter of at least 5 mm. 24 hr after injection.

RESULTS

All strains tested were toxic for mice and induced skin lesions in guinea-pigs. Uninfected yolk sac suspensions never killed mice; they sometimes induced lesions in guinea-pigs but never beyond a dilution of $10^{-1\cdot5}$.

Groups of eggs were inoculated with different amounts of virus; each group was sampled daily by harvesting yolk sacs from two to four live embryos, making a suspension, titrating it in mice and guinea-pigs and estimating the total particles present. Virus suspensions induced measurable lesions in guinea-pigs 4–5 days, and killed mice 1–2 days before the first chick embryo deaths occurred. The results of a representative experiment are given in Table 1. In terms of their effect on mice and guinea-pigs, suspensions contained most toxic material around the mean death time of the group of embryos. Suspensions made from dead embryos induced skin lesions in guinea-pigs but rarely killed mice. A similar pattern was observed for all strains, but the interval between inoculation of embryos and first appearance of toxin, and the time of its maximum titre varied with the strain as well as the dose. For example, when 25 ELD50 of SA2*f* were inoculated, the mean death time of the embryos was 7·5 days and the toxicity for mice was greatest on the seventh day, whereas with 40 ELD50 of LB4 the mean death time was 10·3 days and toxicity was maximum 10 days after inoculation.

Although toxicity increased during the growth cycle, the ratio of elementary bodies to mouse LD50 showed no consistent pattern of change and for all strains

Table 1. *The toxicity of yolk sacs from live eggs harvested at various times from groups of eggs receiving different doses of agent LB4*

Log_{10} egg LD 50 inoculated	Mean death time of group (days)*	Day sample taken	Log_{10} mouse LD 50 per yolk sac sampled	Guinea-pig skin lesion dose† per yolk sac sampled
3·4	7·5	5	< 1	3·3
		6	1·3	4·8
		7	1·4	4·6
		8	1·1	4·3
1·6	10·3	7	< 1	3·1
		8	1·3	3·5
		9	1·4	5·1
		10	1·6	6·1
		11	1·6	4·5
0·3	11·3	9	< 1	3·4
		10	1·3	4·3
		11	1·5	6·9
		12	1·7	5·8

* $\frac{\Sigma(\text{no. embryos dying} \times \text{day after inoculation on which each died})}{\text{total no. embryos dying}}$.

† Reciprocal of highest dilution giving a 5 mm. lesion in guinea-pigs injected intracutaneously expressed as $\log_{10}$.

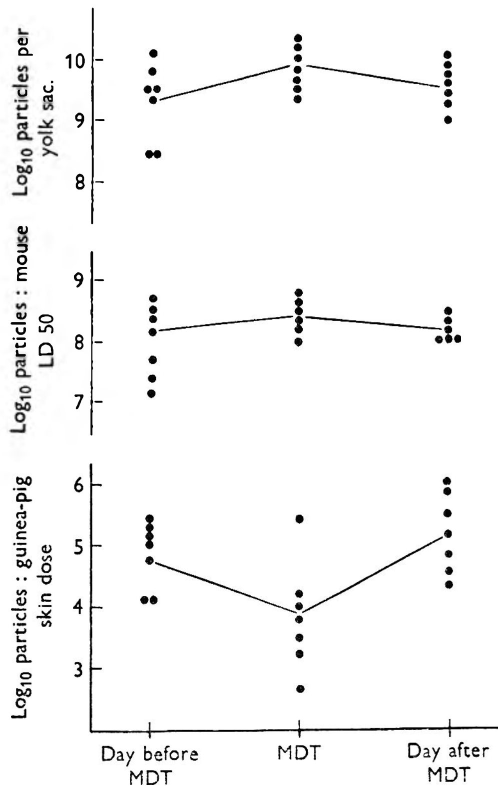


Fig. 1. The total number of particles in a yolk sac and the ratios of total particles: mouse LD 50, and total particles: guinea-pig skin dose around the mean death time (MDT) of groups of infected chick embryos. Six strains were tested, one twice. The lines connect the mean of each group of values.

Table 2. *Toxicity and particle:toxicity ratios of suspensions of TRIC agents that killed mice*

Strain	*Total particles per yolk sac	Mouse LD 50 per yolk sac	Guinea-pig† skin dose per yolk sac	Particles: mouse LD 50	Particles: guinea-pig skin dose
LB 4	8·5	1·3	4·3	7·2	4·2
	8·6	1·0	4·8	7·6	3·8
	8·6	1·0	4·8	7·6	3·8
	8·6	1·1	4·3	7·5	4·3
	8·9	1·0	5·3	7·9	3·6
	9·1	1·1	6·3	8·0	2·8
	9·5	1·3	3·5	8·2	6·0
	9·6	1·3	4·8	8·3	4·8
	9·6	1·4	6·8	8·2	2·8
	9·6	1·6	6·8	8·0	2·8
	10·0	1·5	4·5	8·5	5·5
	10·1	1·4	5·0	8·7	5·0
	10·2	1·6	6·0	8·6	4·2
SA 2	9·7	1·3	4·5	7·4	5·2
	9·8	1·6	4·0	8·2	5·8
G 1	9·3	1·1	4·8	8·2	4·5
	9·6	1·1	4·3	8·5	5·3
	9·8	1·1	6·3	8·7	3·5
	10·0	1·3	6·8	8·7	3·2
	10·2	1·5	6·8	9·0	3·4
LB 4 *f*	8·5	1·1	4·1	7·4	4·4
	9·0	0·7	3·8	8·3	5·2
	9·6	1·1	6·3	8·5	3·3
	10·1	1·7	5·6	8·4	4·5
SA 2 *f*	9·5	1·3	5·5	8·2	4·0
	9·8	1·2	5·0	8·6	4·8
	9·8	1·6	5·5	8·2	4·3
T'ang	9·4	1·0	4·0	8·4	5·4
	10·3	1·9	6·5	8·4	3·8

* All titres expressed in $\log_{10}$.
† Reciprocal of highest dilution giving a 5 mm. lesion.

remained within the same limits. For example, with LB 4, this ratio varied from $1·6 \times 10^7$ to $5·2 \times 10^8$ (Table 2) and fell within these limits with all other strains and the fast-killing variants. The amount of virus necessary to induce a skin lesion in a guinea-pig was always much less than that required to kill a mouse; no great differences were observed between strains. However, for every strain tested, fewer particles were needed to produce a lesion at the mean death time of the eggs than on the day before or the day after (Fig. 1).

DISCUSSION

We tested the toxicity of strains of trachoma and inclusion conjunctivitis isolated in different laboratories, and of fast-killing variant strains. The toxicity of suspensions for mice always reached a maximum at the mean death time of the group of infected eggs, and although this time depended on the strain, no great differences

were found in the ratio of total particles to mouse LD50. With frozen pools of several other strains including T'ang, made at a time likely to give maximum toxicity, Reeve (1964) obtained ratios of particles to mouse LD50 from $2 \cdot 3 \times 10^7$ to $4 \cdot 5 \times 10^8$. Thus all strains tested have given similar results. Furthermore, the toxicity:particle ratio of TRIC agents is similar to that of the meningopneumonitis agent, another member of the psittacosis-lymphogranuloma group (Manire & Smith, 1959).

The results of the guinea-pig skin test also showed no differences between TRIC strains, including the fast-killing variants. Except at the mean death time of the group of infected eggs, an average of 8×10^4 particles of any strain were required to produce a skin lesion 5 mm. in diameter. At the mean death time, however, only 8×10^3 particles were required; a 10-fold difference seen with every strain and only at this time. The mouse test did not reflect this change. Little is known about the lethal action of these agents in mice, still less about the causes of the skin lesion in guinea-pigs. The guinea-pig test may measure a toxin different from that killing mice. It is also possible that suspensions made at the mean death time of the chick embryos contain products of damaged yolk sac cells that contribute to the skin lesion but do not kill mice under these conditions. Whether or not TRIC strains were fast-killing in eggs, no differences in toxicity were detected in mice or guinea-pigs. Differences in the amount of toxin per particle cannot therefore explain the strains' differing virulence for chick embryos.

REFERENCES

COLLIER, L. H. & SOWA, J. (1958). Isolation of trachoma virus in embryonate eggs. *Lancet*, i, 993.

DULBECCO, R. & VOGT, M. (1954). Plaque formation and isolation of pure lines with poliomyelitis virus. *J. exp. Med.* **99**, 2.

GEAR, J. H. S., GORDON, F. B., JONES, B. R. & BELL, S. D. Jr. (1963). Nomenclature of isolates of virus from trachoma and inclusion blennorrhoea. *Nature, Lond.* **197**, 26.

JONES, B. R. (1961). TRIC virus infections in London. *Trans. Ophthal. Soc. U.K.* **81**, 367.

MANIRE, G. P. & SMITH, K.O. (1959). Quantitative studies on meningopneumonitis virus. *J. Bact.* **78**, 523.

MURRAY, E. S., BELL, S. D. Jr., HANNA, A. T., NICHOLS, R. L. & SNYDER, J. C. (1960). Studies on trachoma. 1. Isolation and identification of strains of elementary bodies from Saudi Arabia and Egypt. *Amer. J. Trop. Med. Hyg.* **9**, 116.

REED, L. J. & MUENCH, H. (1938). A simple method of estimating fifty per cent endpoints. *Amer. J. Hyg.* **27**, 493.

REEVE, P. (1964). Trachoma viruses isolated in the United States. VII. Related toxicity of different strains for white mice. *Proc. Soc. exp. Biol., N.Y.* **116**, 373.

REEVE, P. & TAVERNE, J. (1962). A simple method for total particle counts of trachoma and inclusion blennorrhoea virus. *Nature, Lond.* **195**, 923.

REEVE, P. & TAVERNE, J. (1963). Observations on the growth of trachoma and inclusion blennorrhoea viruses in embryonate eggs. *J. Hyg., Camb.* **61**, 67.

T'ANG, F. F., CHANG, H. L., HUANG, Y. T. & WANG, K. C. (1957). Studies on the etiology of trachoma with special reference to isolation of the virus in chick embryo. *Chin. med. J.* **75**, 49.

J. gen. Microbiol. (1964), 37, 277–284
Printed in Great Britain

Growth in the Chick Embryo of Strains of Trachoma and Inclusion Blennorrhoea Virus of Differing Virulence

By JANICE TAVERNE, W. A. BLYTH AND P. REEVE
M.R.C. Trachoma Research Unit, Lister Institute of Preventive Medicine, Chelsea Bridge Road, London, S.W.1

SUMMARY

The growth in the chick embryo yolk sac of trachoma and inclusion conjunctivitis (TRIC) strains which differ in virulence for the chick embryo was measured in terms of ELD50 inclusion forming units in HeLa cells and total particles. Observed differences in rates of growth are consistent with the assumption that greater virulence depends on a higher rate of multiplication in the chick embryo. All strains were equally labile when heated at 37° *in vitro* but only the more virulent kill chick embryos at 37°.

INTRODUCTION

The capacity of a micro-organism to kill is covered by the term virulence, and virulence may be determined by toxigenicity, invasiveness, or many other factors and is qualified by the host in which it is measured (*Topley & Wilson's Principles*, 1964). Strains of trachoma and inclusion conjunctivitis (TRIC) agents differ in ability to kill chick embryos when inoculated into the yolk sac, some strains killing more quickly than others when the same number of infectious units are injected (Reeve & Taverne, 1963). These faster killing strains may be regarded as more virulent for the chick embryo. We are interested in the factors determining the virulence of TRIC agents for the chick embryo. Previously, we suggested that differences in virulence might depend on differences in toxicity (Reeve & Taverne, 1963), but subsequently found that elementary bodies of strains of differing virulence contained the same amount of toxin (Taverne, Blyth & Reeve, 1964). Like Jawetz & Hanna (1960) we concluded from the analysis of dose–response curves that all strains tested multiplied at the same rate. However, eggs infected with a small dose of more virulent strains die 2–3 days earlier than those infected with a similar dose of less virulent strains, yet in each instance the yolk sacs at death contain 10^9–10^{10} elementary bodies (Reeve & Taverne, 1963; Taverne, Blyth & Reeve, 1964). This implies that the growth pattern of the more virulent strains must differ in some way. For instance, the lag before exponential growth or the interval between the end of exponential growth and the death of the embryo might be shorter, or the growth rate might be faster. Jawetz, Hanna, Chino & Zichosch (1962) measured growth rates directly in eggs and concluded that they did not vary significantly with the strains tested. Since minor variations in the mode of growth might account for the differences observed, we investigated the growth in the chick embryo yolk sac of strains of differing virulence by titrating virus in eggs and in HeLa cells, and estimating the total number of elementary bodies.

METHODS

TRIC agents are named according to the system proposed by Gear, Gordon, Jones & Bell (1963). Their original names used in this paper are given in brackets. Fast-killing variants are suffixed *f*.

TRIC/China/Peking-2/OT (T'ang, Chang, Huang & Wang 1957).

TRIC/2/SAU/HAR-2/OT (SA 2) (Murray *et al.* 1960) received from Miss L. Hanna, University of California Medical Center, San Francisco. The variant, SA 2*f*, derives from material received from Dr S. Bell, School of Public Health, Harvard University.

TRIC/ /WAG/MRC-1/OT (G 1) (Collier & Sowa, 1958).

TRIC/ /WAG/MRC-187/OT (G 187) isolated in the Gambia, British West Africa, from a patient with trachoma.

TRIC/ /WAG/MRC-062/OT (G 062) isolated in the Gambia, British West Africa, from a patient with trachoma.

TRIC/ /GB/MRC-4/ON (LB 4) (Jones, 1961) and the variant LB 4*f*.

TRIC/ /USA-Cal/Cal-1/OT (BOUR) (Hanna, Jawetz, Thygeson & Dawson, 1960).

TRIC/ /GB/MRC/1/G (LB 1) (Jones, Collier & Smith, 1959).

Diluent: phosphate buffered saline (Dulbecco & Vogt, 1954) containing streptomycin sulphate 1000 μg./ml.

Virus suspensions: 10 % or 20 % (w/v) suspensions were made by shaking infected yolk sacs in diluent and then discarding the membranes.

Virus titrations were done in 7-day chick embryos kept at 35° and candled daily. The tests were terminated on the twelfth day after inoculation. Titres are expressed in terms of 50 % lethal dose for eggs (ELD 50; Reed & Muench, 1938). Specificity of death, when doubtful, was determined by examining yolk sac smears for elementary bodies. Virus was titrated in HeLa cells (Furness, Graham & Reeve, 1960) and titres are expressed in terms of inclusion-forming units (IFU).

Particle counts were made by the dark-field method of Reeve & Taverne (1962) except that treatment with trypsin was omitted and suspensions were purified by centrifugation at 8000 *g* for 20 min. in M-KCl.

RESULTS

Growth curves. The growth of 6 strains in the chick embryo yolk sac at 35° was investigated. For each strain, fifty 7-day eggs were injected with a dose of virus chosen to kill the embryos as late as possible. Daily, yolk sacs were taken from 3 live embryos, pooled and shaken with a measured volume of diluent; the suspension was titrated in eggs and HeLa cells, and the total number of particles estimated. Growth curves plotted to show the increase in infectious virus during the course of infection are given for 4 strains in Fig. 1. The total particle counts for all 6 strains and the elementary body: infectious virus ratios are presented in Table 1. With no strain was virus detected before the second day of infection. From the second day the number of ELD 50 doses increased exponentially to reach a maximum at the mean death time of each group of eggs. During the last 4 days of growth elementary bodies were sufficiently numerous to be counted by the dark ground technique, and, with the more virulent strains, enough virus was present by the fourth day to be

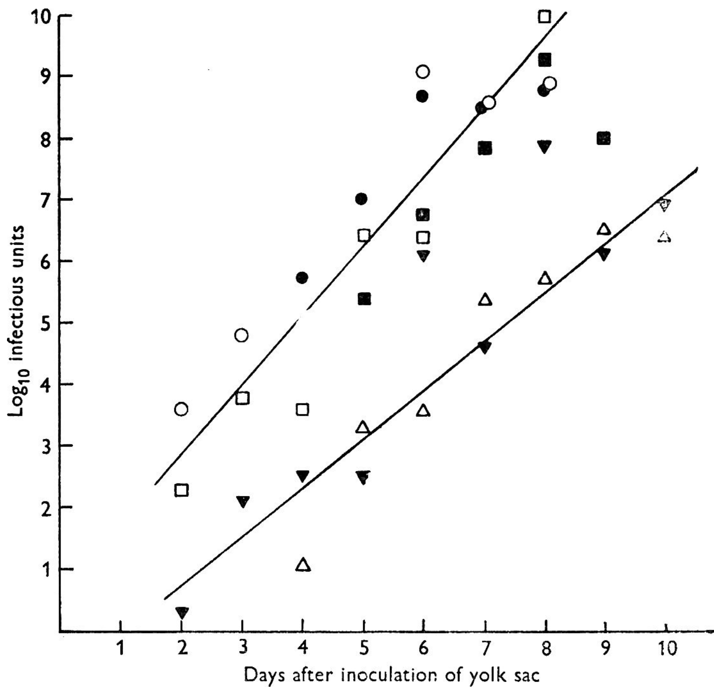


Fig. 1. Growth in yolk sac of two virulent TRIC agents and two less virulent strains. Infecting doses: T'ang $10^{1 \cdot 3}$ ELD50, LB4 $10^{1 \cdot 6}$ ELD50, SA2*f* $10^{1 \cdot 7}$ ELD50, SA2 $10^{1 \cdot 4}$ ELD50. □, T'ang ELD50; ■, T'ang IFU; ○, SA2*f* ELD50; ●, SA2*f* IFU; △ LB4 ELD50; ▼, LB4 ELD 50. The lines drawn are best fits derived by a method of least recorded squares.

Table 1. *The growth of six TRIC agents in the chick embryo yolk sac*

Doses inoculated and mean death time are shown in Table 2.

Particle numbers for egg LD 50 (ELD 50) or inclusion-forming units (IFU) per yolk sac (YS) are recorded as $\log_{10}$.

	Fast-killing strains								
Days after inoculation	T'ang. Particles			SA 2*f*. Particles			LB 4*f*. Particles		
	Per YS	Per ELD 50	Per IFU	Per YS	Per ELD 50	Per IFU	Per YS	Per ELD 50	Per IFU
4	—	—	—	7·2	—	2·7	—	N.D.	—
5	8·3	1·9	2·0	7·9	—	1·0	7·3	N.D.	1·0
6	7·6	1·2	0·8	9·8	0·7	1·1	8·9	N.D.	2·6
7	9·4	—	1·6	9·6	1·0	1·1	9·7	N.D.	1·6
8	10·3	0·3	1·2	9·9	1·0	1·0	—	N.D.	—
9	9·5	—	1·5	—	—	—	—	N.D.	—

			Slow-killing strains			
Days after inoculation	G 1. Particles		SA 2. Particles		LB 4. Particles	
	Per YS	Per ELD 50	Per YS	Per ELD 50	Per YS	Per ELD 50
4	—	N.D.	—	—	7·9	5·4
5	—	N.D.	—	—	8·3	5·8
6	—	N.D.	8·0	4·4	8·9	2·8
7	8·2	N.D.	7·9	2·5	8·8	4·2
8	9·2	N.D.	9·3	3·6	9·5	1·6
9	9·2	N.D.	9·7	3·2	10·1	4·0
10	9·2	N.D.	9·0	4·1	10·2	3·3
11	10·4	N.D.	10·0	—	10·1	—

detected as inclusion-forming units in HeLa cells. From then on the infectivity of suspensions was the same in HeLa cells as in eggs, and growth curves measured in IFU were similar to those measured in ELD50. The less virulent strains did not form enough inclusions to be counted except at the end of the growth when some suspensions with very large amounts of virus infective for eggs (10^7 ELD50 per yolk sac) contained a small proportion of inclusion-forming units (about 10^5 IFU per yolk sac). The slopes of the growth curves differed, virulent strains apparently increasing at a faster rate than the less virulent (Table 2).

Table 2. *Rates of increase of six TRIC strains growing in the chick embryo yolk sac*

	Strain	Dose inoculated log_{10} ELD50	Mean death time (days)	Rates of increase log_{10}/day ELD50	IFU	Total particles
Fast-killing strains	T'ang	1·3	8·0	1·3	1·3	1·3
	SA 2*f*	1·7	6·6	1·8	1·8	*0·6–1·3
	LB 4*f*	3·5	7·3	N.D.	1·3	1·3
Slow-killing strains	G 1	2·3	9·8	N.D.	—	0·7
	SA 2	1·4	10·0	0·9	—	0·7
	LB 4	1·6	10·3	0·8	—	0·7

* Wide scatter obtained, limits quoted.

Table 3. *Titration of yolk sac suspensions in chick embryos at 35° and 37°*

	Strain	ELD50 per ml. at 35°	ELD50 per ml. at 37°
Fast-killing strains	T'ang	4·8	4·8
	SA2*f*	7·0	6·6
	LB4*f*	6·9	6·6
	LB1	5·6	5·6
Slow-killing strains	G1	3·0	Nil
	SA2	5·8	4·7
	SA2*	5·6	3·9
	LB4	2·7	Nil
	G 062	5·2	Nil
	G187	4·5	0·5
	BOUR	4·6	Nil

* Calculated from data of Bell *et al.* (1963).

Although similar numbers of ELD50 doses were inoculated, the more virulent strains always yielded more infective virus. However, at the mean death time of each group of embryos, yolk sacs infected with any strain contained about the same number of elementary bodies. During the latter part of growth, when elementary bodies could be counted, the ratio of total particles:ELD50 was on average 10 for more virulent strains and 5000 for less virulent strains (Table 1). One explanation for this finding is that the less virulent strains are more susceptible to heat. Accordingly, tests were made of the behaviour of the strains at different temperatures.

Titrations at different temperatures. For each strain one set of dilutions was used to inoculate two sets of eggs, which were then incubated at 35° and 37° respectively.

Incubator temperatures were controlled to $\pm 0 \cdot 5^{\circ}$ and checked by recording thermometers. A high ambient humidity was maintained. The infectivity of the more virulent strains was measurable at 37° and titres, although lower, were within threefold of those at 35° (Table 3). By contrast, the less virulent strains, except SA 2, failed to kill chick embryos at 37°; since their titres at 35° ranged from $10^{2 \cdot 7}$ to $10^{5 \cdot 2}$ ELD50 per ml. there was at least 1000-fold difference in titre. The titre of strain SA 2 at 37° was ten-fold lower than at 35°. Differences in virulence were thus reflected by differences in killing power at 37°; only the more virulent strains—and SA 2—killed embryos at 37°.

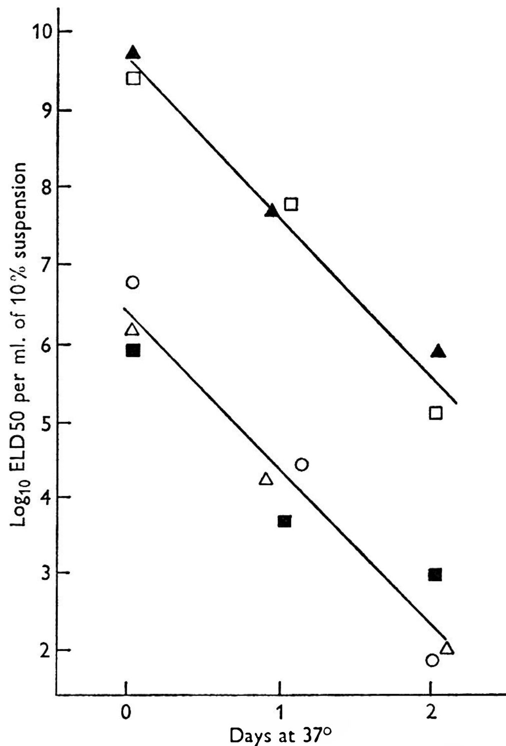


Fig. 2. Inactivation *in vitro* of six TRIC agents at 37°. △, SA 2*f* pool 1; ▲, SA 2*f* pool 2; □, LB 4*f*; ○, G 1; ■, LB 4.

Heat inactivation. Ten per cent (w/v) suspensions of infected yolk sacs in diluent were kept in a water bath at 37° and titrated at intervals for infectivity. The strains tested did not vary significantly in rate of inactivation (Fig. 2), all decreasing in infectivity by 100–300 ELD50 per day.

DISCUSSION

We investigated several TRIC agents to determine whether differences in growth rates in the chick embryo were related to virulence. Variations in the initial lag, before exponential growth began, were insufficient to explain the difference of 2–3 days observed in the time to death of embryos infected with fast- and slow-

killing strains. Furthermore, there was no interval between the attainment of maximum virus titres and embryo death. When yolk sacs contained most virus, measured in terms of ELD50, IFU or total particles, the infected embryos began to die. Toxin, titrated in mice or in guinea-pig skin, also reached its maximum at this time (Taverne *et al.* 1964).

We considered the significance of the small differences in growth rate observed during the exponential phase of multiplication. Theoretically, with a small infecting dose, a strain that shows a daily twentyfold increase in the number of particles would produce 10^9–10^{10} particles 7–8 days after inoculation, if there were no lag before exponential increase. With a daily tenfold increase, this number of particles would be produced in 10 days. Thus a difference of only twofold in the rate of multiplication would account for a difference of 2–3 days in the time at which the strains attained maximum titres of 10^9–10^{10}. The formal demonstration of a statistically significant difference in the slopes shown in Fig. 1 would require a prohibitive number of replicate experiments. However, the observed difference, if real, is sufficient to account for the difference in killing time. It is understandable that small differences in growth rate have not been considered significant (Jawetz & Hanna, 1960; Jawetz *et al.* 1962; Reeve & Taverne, 1963) because titration techniques used are insufficiently precise. Micro-organisms of the psittacosis-lymphogranuloma group are known to have a complicated life cycle, initial forms dividing to produce elementary bodies. The differences in growth rate observed need not only imply differences in the rate of fission; they could result from variations in the mode of division—for instance in the number of elementary bodies derived from each initial form. Again, the growth curves are the results of many cycles of infection, and if strains differed in the efficiency with which they entered cells, a more invasive strain would appear more virulent without necessarily having a different intracellular growth cycle.

Throughout growth the virulence, in terms of organisms per ELD50, was unchanged, in both the less virulent strains with an ELD50 of 5000 and the more virulent strains with an ELD50 of 10 organisms. In addition, the ratio of elementary bodies to infective virus was constant for all strains, suggesting that there was no progressive inactivation *in vivo*.

With some microbes, the more virulent strains are characterized by capacity to grow over a wider temperature range than the less virulent. Thus Dubes & Wenner (1957) found that virulent strains of poliovirus grew more efficiently at 39° than attenuated strains. Nizamuddin & Dumbell (1961) showed that variola major virus produces pocks on the chick chorioallantois at 39°, whereas the less virulent variola minor virus does not. Bedson & Dumbell (1961) discovered that the virulence of pox virus strains in terms of ability to kill chick embryos is related to the highest temperature ('ceiling temperature') at which they will grow. We did not determine the ceiling temperatures of our strains, but found that only the more virulent strains—and SA2—killed chick embryos at 37°. Our results with strain SA2 are similar to those of Bell, Murray, Carroll & Snyder (1963). This strain killed chick embryos at 37° but there were fewer ELD50 doses than at 35°. The figures given by Bell and co-workers show that embryos inoculated with a given dose of SA2 and incubated at 37° regularly died 2–3 days earlier than those incubated at 35°, as we also observed. The effects of a higher temperature of incubation on SA2 were thus

twofold: titres were lower and chick embryos died more quickly. Only with strain SA 2 was this shortening in time of response observed. With more virulent strains dose-response curves plotted from titrations at 35° were similar to those obtained from titrating at 37°. It seems likely that strain SA 2 is heterogeneous, containing particles that differ in ability to kill chick embryos at different temperatures. Our results emphasize the importance of controlling incubator temperatures within narrow limits, as already stressed by Bell *et al.* (1963), since suspensions usually contained fewer ELD 50's at 37° and in many cases failed to kill embryos.

As with the pox strains tested by Bedson & Dumbell (1961), differences in the ability to grow at higher temperatures do not appear to depend on differences in the rate of inactivation by heat, all strains being equally labile when heated at 37° *in vitro.* Reeve & Taverne (1963) established that strains of relatively greater virulence for the chick embryo can also form inclusions in HeLa cells and that some of them can multiply in the mouse brain. We have now added another character associated with virulence, namely that virulent strains kill chick embryos at 37°, whereas less virulent strains do not. Our results, moreover, are consistent with the assumption that greater virulence is dependent on a higher rate of multiplication in the chick embryo.

We are grateful to Dr L. H. Collier for his help and criticism.

REFERENCES

BEDSON, H. S. & DUMBELL, K. R. (1961). The effect of temperature on the growth of pox viruses in the chick embryo. *J. Hyg., Camb.* **59**, 457.

BELL, S. D. JUN., MURRAY, E. S., CARROLL, T. J. & SNYDER, J. C. (1963). Studies on trachoma. III. Comparison of virus infectivity for man and chick embryos. *Amer. J. trop. Med. Hyg.* **12**, 902.

COLLIER, L. H. & SOWA, J. (1958). Isolation of trachoma virus in embryonate eggs. *Lancet*, i, 993.

DUBES, G. R. & WENNER, H. A. (1957). Virulence of polioviruses in relation to variant characteristics distinguishable on cells in vitro. *Virology*, **4**, 275.

DULBECCO, R. & VOGT, M. (1954). Plaque formation and isolation of pure lines with poliomyelitis virus. *J. exp. Med.* **99**, 2.

FURNESS, G., GRAHAM, D. M. & REEVE, P. (1960). The titration of trachoma and inclusion blennorrhoea viruses in cell cultures. *J. gen. Microbiol.* **23**, 613.

GEAR, J. H. S., GORDON, F. B., JONES, B. R. & BELL, S. D. JUN. (1963). Nomenclature of isolates of virus from trachoma and inclusion blennorrhoea. *Nature, Lond.* **197**, 26.

HANNA, L., JAWETZ, E., THYGESON, P. & DAWSON, C. (1960). Trachoma viruses isolated in the United States: I. Growth in embryonated eggs. *Proc. Soc. exp. Biol., N.Y.* **104**, 142.

JAWETZ, E. & HANNA, L. (1960). Trachoma viruses isolated in the United States: II. Assay of egg infectivity and stability. *Proc. Soc. exp. Biol., N.Y.* **105**, 207.

JAWETZ, E., HANNA, L., CHINO, S. & ZICHOSCH, J. (1962). Trachoma viruses isolated in the United States. V. Growth rates and drug inhibition patterns in embryonated eggs. *Proc. Soc. exp. Biol., N.Y.* **109**, 205.

JONES, B. R. (1961). TRIC virus infections in London. *Trans. ophthal. Soc. U.K.* **81**, 367.

JONES, B. R., COLLIER, L. H. & SMITH, C. H. (1959). Isolation of virus from inclusion blennorrhoea. *Lancet*, i, 902.

MURRAY, E. S., BELL, S. D. JUN., HANNA, A. T., NICHOLS, R. L. & SNYDER, J. C. (1960), Studies on trachoma. 1. Isolation and identification of strains of elementary bodies from Saudi Arabia and Egypt. *Amer. J. trop. Med. Hyg.* **9**, 116.

Nizamuddin, N. & Dumbell, K. R. (1961). A simple laboratory test to distinguish the virus of smallpox from that of alastrim. *Lancet*, i, 68.

Reed, L. J. & Muench, H. (1938). A simple method of estimating fifty per cent endpoints. *Amer. J. Hyg.* 27, 493.

Reeve, P. & Taverne, J. (1962). A simple method for total particle counts of trachoma and inclusion blennorrhoea viruses. *Nature, Lond.* **195**, 923.

Reeve, P. & Taverne, J. (1963). Observations on the growth of trachoma and inclusion blennorrhoea viruses in embryonate eggs. *J. Hyg., Camb.* **61**, 67.

T'ang, F. F., Chang, H. L., Huang, Y. T. & Wang, K. C. (1957). Studies on the etiology of trachoma with special reference to isolation of the virus in chick embryo. *Chin. med. J.* 75, 49.

Taverne, J., Blyth, W. A. & Reeve, P. (1964). Toxicity of the agents of trachoma and inclusion conjunctivitis. *J. gen. Microbiol.* 37, 271.

Topley and Wilson's Principles of Bacteriology and Immunity (1964). 5th ed. Ed. by G. S. Wilson & A. A. Miles. London. Edward Arnold and Co.

J. gen. Microbiol. (1964), 37, 285–292
Printed in Great Britain

The Relation of Erythritol Usage to Virulence in the Brucellas

BY A. E. WILLIAMS, J. KEPPIE AND H. SMITH
Microbiological Research Establishment, Porton, Salisbury, Wiltshire

(27 *July* 1964)

SUMMARY

Erythritol stimulated the growth of virulent strains of *Brucella abortus* more than that of attenuated strains. No similar correlation existed between virulence and response to erythritol with strains of *B. melitensis* and *B. suis*; these were stimulated indiscriminately by erythritol.

Erythritol was not detected in extracts of bovine white blood cells and hence is unlikely to be connected with the primary invasive process of intraphagocytic survival and growth of *B. abortus*. However, ultrafiltrates from extracts of bovine white blood cells stimulated the growth of *B. abortus* in laboratory media.

INTRODUCTION

The presence of erythritol—a growth stimulant for brucellas—in susceptible tissues appears to explain the localization of brucellas in the placenta and male genitalia of susceptible animal species; those animal species which do not suffer intense placentitis in brucellosis had no detectable erythritol in their placentas (Smith *et al.* 1962; Pearce *et al.* 1962; Williams, Keppie & Smith, 1962; Keppie, Williams, Witt & Smith, 1965). In these studies, single virulent strains of *Brucella abortus*, *B. melitensis* and *B. suis* were used. The effect of erythritol on the growth of a number of strains has been investigated to see whether a connexion exists between erythritol usage and virulence. McCullough & Beal (1951) had already shown that the growth of four different virulent strains of each Brucella species was stimulated by erythritol when it was present as the sole carbon and energy source in a simple basal medium. In the present studies, we have examined the effect of erythritol on the growth of several virulent and avirulent strains of Brucella in media containing adequate glucose. Some correlation was found between the virulence of various strains of *B. abortus* and their growth response to erythritol. These findings could not be extended to strains of *B. melitensis* and *B. suis* but they prompted the investigation reported in this paper.

The ability of various strains of *Brucella abortus* to survive phagocytosis and multiply within bovine phagocytes is correlated with their virulence and appears to rely to some extent on protective cell-wall materials which may be produced more by virulent than by avirulent cells (Smith & Fitzgeorge, 1964; Macrae & Smith, 1964). However, avirulent cells produce some protective material and a factor which may also contribute to the different behaviour of avirulent and virulent strains on phagocytosis might be a superior ability of the latter to exploit the growth conditions of the host's phagocytes, while the bactericidal mechanisms of the latter are neutralized by the cell-wall materials. This hypothesis would be supported if the phagocytes contained small amounts of erythritol which could stimulate the growth of virulent rather than avirulent strains of *B. abortus*. Hence, extracts of white blood cells and other constituents of blood were examined for the presence

of small amounts of erythritol and for the ability to stimulate the growth of virulent and avirulent strains of *B. abortus* in laboratory media.

METHODS

Strains of Brucella. The virulence of the Brucella strains was determined in guinea pigs as follows; a dose of 3×10^6 for *Brucella abortus* and *B. melitensis*, and 1×10^3 for *B. suis* was injected intramuscularly into guinea pigs (400 ± 50 g.). Three weeks later the average count in the spleens of these animals was determined by the culture of suitable dilutions of macerated spleen. The strains were designated virulent when the spleen counts were greater than 3×10^4, attenuated when greater than 1×10^3 and less than 3×10^4, and avirulent when less than 1×10^3 (cf. Table II of Smith & Fitzgeorge, 1964):

B. abortus strains	Virulent	2308, BL 297, B 265, BF 79, A 5, 544 and 1503
	Attenuated	205/19, S 19, 58/20 and 45/20
	Avirulent	11, 99, 45/0
B. melitensis strains	Virulent	6015, 1374 and ether
	Attenuated	61/115 and Rev. 1
	Avirulent	B 115, 61/118
B. suis strains	Virulent	KG 25, 8973/56, 1330, 15 LA, and 63/171
	Attenuated	63/166, 61/59, 61/66, and Thomsen
	Avirulent	63/260 and 61/60

Erythritol was supplied by G. T. Gurr Ltd. and recrystallized from moist acetone.

Tests for ability of materials to stimulate the growth of Brucella in vitro

(*a*) *Viable count method.* The method has been described previously (Williams *et al.* 1962). The basal medium was a mixture of tryptic digest meat broth (1 part) and isotonic saline (2 parts); the inoculum was 100 organisms/ml.

(*b*) *Turbidimetric methods.* Shake cultures in cuvette-flasks were used (Copeland, 1955). Optically matched Pyrex test-tubes were fixed to the sides of 50 ml. conical flasks. Medium, inoculated with the test organism (see below) was distributed into the cuvette-flasks (10 ml. per flask). Experimental flasks received erythritol in Locke solution (0·2 ml.). Control flasks received Locke solution (0·2 ml.). The flasks were incubated on a horizontal shaker at 37°. Strains requiring added CO_2 for growth were incubated in sealed aluminium boxes gassed with air containing 5% CO_2. When turbidity readings were required the flasks were turned through approximately 120°, thus transferring the culture to the side-arm, and turbidimetric readings were obtained directly in the Coleman spectrophotometer. The flasks were then returned to the shaker when subsequent readings were required for the preparation of growth curves. The optical densities of duplicate flasks varied by less than 3%. When required (see Table 2) regression coefficients for the log phase of growth were calculated from the curves between 17 and 25 hr and the ratios of the growth rates with and without erythritol were obtained.

Two media were used in this work.

Medium (1): a mixture of tryptic digest meat broth with Locke solution (1 + 1 by vol.) with an inoculum of $1–2 \times 10^6$/ml. Turbidimetric readings were made at 570 mμ.

Medium (2): a modified form of that used by Rode, Oglesby & Schuhardt (1950).

A glutamate-free modification of this medium (Anderson & Smith, 1965) would not support the growth of avirulent strains of *Brucella abortus*; hence 100 μg. L-glutamic acid/ml. was added. The modified medium differed from that of Rode *et al.* (1950) as follows. D-glucose 7·0 mg./ml. (instead of 4·0 mg./ml.); ammonium sulphate 1·35 mg./ml. (instead of nil); L-glutamic acid 100 μg./ml. (instead of 3000 μg. DL-glutamic acid/ml.). A concentrated (4 ×) stock solution, minus the biotin, glucose and glutamic acid, was stored at $-20°$. A supply of medium for 2 weeks was prepared from the stock solution by dilution, addition of biotin and filtration through a 'Millipore' filter. The filtered solution was stable at 0–4° for 14 days. Glucose and glutamate were added before each experiment and the medium refiltered. The medium was inoculated with 2×10^7/ml. and extinction measurements on cultures were made at 430 mμ.

Estimation of erythritol. The method has been described by Williams *et al.* (1962).

Preparation of cell extracts from bovine blood for biological and chemical analysis

Differential centrifugation of blood yielded large quantities of white blood cells which were purified from red blood cells. The white blood cells were disrupted ultrasonically and the ultrafiltrates of the extracts were examined. Red blood cells were also obtained and disrupted by freezing and thawing.

Citrated (0·25 %) bovine blood (10 l.) was centrifuged for 60 min. at 2° in an M.S.E. Major centrifuge. The plasma was removed and a 200 ml. sample frozen at $-20°$; a 200 ml. sample of the red blood cells also was frozen at $-20°$. The 'buffy coat' (300–750 ml.) containing the majority of white blood cells was removed by pipette and centrifuged at 27,000 rev./min. for 60 min. in the 30 rotor of a Spinco ultracentrifuge. The plasma from the deposit was decanted revealing the white blood cells as a coherent 'skin' (2–5 mm. thick) on the surface of the remaining erythocytes. This skin was tipped from each tube on to a microscope slide ($3 \times 1\frac{1}{2}$ in.), and the slide inverted on a white enamel tray. Careful removal of the slide showed a contaminating layer of erythrocytes on the lower surface of the leucocyte skin. By gently drawing the edges of two other slides across this exposed surface (beginning at the centre and moving in opposite directions) the majority of these erythrocytes were removed. These manipulations were carried out at 0–4°. The 'pure' leucocyte preparation was frozen at $-20°$. A yield of $2–4 \times 10^{10}$ leucocytes was obtained from 10 l. of bovine blood. Haemocytometer counts showed that samples prepared by this method had W.B.C.: R.B.C. ratios which varied from 2:1 to 1:5. Dye-exclusion tests with eosin indicated that 50–75 % of leucocytes in the final sample were viable.

The three fractions were treated as follows:

Leucocyte fraction. This was thawed and homogenized by maceration in an M.S.E. overhead macerator with the minimum quantity (about 20 ml.) of isotonic phosphate saline buffer (NaCl 7·5, Na_2HPO_4 1·42, KH_2PO_4 1·36 g./l.; pH 7·4 with 40 %, w/v, NaOH). The cooled cell suspension was stirred magnetically and treated ultrasonically for 15 min. with the M.S.E. Mullard ultrasonic disintegrator as described by Smith, Keppie, Cocking & Witt (1960). Haemocytometer counts showed that 99·9 % of the leucocytes had disintegrated. The extract was centrifuged at 27,000 rev./min. for 60 min. int he 30 rotor of a Spinco ultracentrifuge. The supernatant fluid was ultrafiltered at 0–2° and the ultrafiltrate stored at $-20°$.

Erythrocyte fraction. This (1×10^{10} red cells/ml. disrupted by the freezing) was thawed and centrifuged at 27,000 rev./min. for 60 min. in the 30 rotor of a Spinco ultracentrifuge. Diffusates, prepared by dialysing the supernatant fluid (200 ml.) against de-ionized water (3×400 ml. over 3 days), were freeze dried. To estimate the total amount of diffusible material in the extracts (so that freeze-dried diffusates could be redissolved at a strength ≡ the original extracts) the losses in dry weight (105°, 16 hr) of small samples of the extracts were obtained after exhaustive dialysis.

Plasma. A diffusate was obtained from the plasma by treating it as described for the extract of erythrocytes.

RESULTS

The effect of erythritol on the growth of strains of Brucella abortus after 24 hr in a broth medium in vitro

The results in Table 1 show that there was some correlation between degree of virulence for guinea pigs and enhancement of growth by erythritol in medium (1), i.e. tryptic digest meat broth + Locke solution (1 + 1). At erythritol concentrations higher than those shown in Table 1 (2–10 μmole/ml.) some stimulation of the growth of the relatively avirulent strains was observed but the degree of stimulation was smaller than that occurring with the more virulent strains.

Table 1. *The relation between the virulence of Brucella abortus strains and growth enhancement by erythritol*

	Erythritol concentration	
Strain	1 μmole/ml.	0·1 μmole/ml.
Virulent		
2308	90*	36
BL 297	88	39
B 265	59	30
BF 79	55	15
A 5	49	18
544	47	19
1503	39	17
Attenuated		
205/19	24	5
45/20	3	−15
S 19	−2	−2
58/20	−7	−2
Avirulent		
11	20	3
99	0	−1
45/0	6	6

* Growth enhancement by erythritol in medium (1), i.e. tryptic digest meat broth + Locke solution (1 + 1) after 24 hr incubation measured as

$$\frac{\text{Optical density of (sample} - \text{control)}}{\text{Optical density of control}} \times \frac{100}{1}.$$

All strains grew at approximately the same rate in the basal medium. Results are averages of several experiments.

The effect of erythritol on the growth rate of strains of Brucella abortus in a defined medium

The growth rates of various strains of *Brucella abortus* were measured in medium (2) with and without erythritol. The weighted means of the ratios of the growth rates from several experiments (Table 2) again indicated the greater response of the virulent strains to erythritol.

Table 2. *Growth rate response to erythritol by Brucella abortus strains in a defined medium (medium (2))*

Strain	Weighted mean*
Virulent	
BL 297	1·82
544	1·47
Attenuated	
205/19	1·01
S 19	0·96
58/20	1·11
Avirulent	
11	1·03
99	1·03
45/0	1·22

All strains grew at approximately the same rate in the basal medium.

$* \ \frac{\text{Gradient log phase with erythritol (2 } \mu\text{mole/ml.)}}{\text{Gradient log phase without erythritol}}$

The growth response to erythritol of strains of Brucella melitensis and B. suis of differing virulence

The effect of erythritol on the growth of strains of *Brucella melitensis* and *B. suis* was investigated under two sets of growth conditions: (1) low erythritol concentrations and small inoculum of organisms—growth being measured by colony count; and (2) high erythritol concentration and large inoculum of organisms—growth being measured turbidimetrically. The results in Table 3 show that all the *B. melitensis* and *B. suis* strains were stimulated to a greater or lesser extent by erythritol irrespective of their virulence for the guinea pig.

The erythritol content of fractions from bovine blood and their growth-promoting activity for Brucella abortus

Diffusates or ultrafiltrates prepared from white blood cells, red blood cells and plasma obtained from several batches of bovine blood were examined chemically for the presence of erythritol. Parallel biological tests were also carried out to see whether these extracts stimulated the growth of virulent and avirulent strains of *Brucella abortus* in a broth medium. The results are shown in Table 4. Erythritol was not detected ($< 5\mu$g./ml. extract) in any of the leucocyte, erythrocyte or plasma samples. However, the four leucocyte extracts, even when considerably diluted, caused significant stimulation of the growth of virulent and avirulent strains of *B. abortus*. In the same experiments, erythritol stimulated only the virulent strains (544 and 2308) and an equivalent amount of glucose had no effect on any

Table 3. *The growth response to erythritol of strains of Brucella melitensis and B. suis*

Strain	Growth enhancement by erythritol measured: By colony counts at 27 hr*	By turbidimetric readings at 22 hr†
B. melitensis		
Virulent		
6015	2·5‡	1·7‡
1374	1·7	4·4
Ether	4·0	5·1
Attenuated		
61/115	3·8	4·3
Rev 1	3·3	—
Avirulent		
B. 115	—	4·2
61/118	3·6	—
B. suis		
Virulent		
KG 25	1·2	1·4
8973/56	1·6	1·6
1330	1·4	2·4
15 LA	1·5	2·4
63/171	—	2·0
Attenuated		
63/166	—	1·1
61/59	—	1·2
61/66	—	1·2
Thomsen	—	5·5
Avirulent		
63/260	—	1·4
61/60	—	1·9

* Medium: tryptic digest meat broth (1 vol.) and isotonic saline (2 vol.); inoculum 100 organisms per ml.; erythritol 0·065 μmole/ml.

† Medium (2) was used; inoculum 2×10^7/ml.; erythritol 2 μmole/ml.

‡ These results are the means of several similar experiments measured as

$$\frac{\text{population of treated sample}}{\text{population of control}}.$$

Table 4. *The growth-enhancing effect on Brucella abortus of diffusates, from extracts of the white blood cells and other fractions of bovine blood, all with an erythritol content of less than 5 µg./ml.*

White blood cell diffusates: W.B.C. count per ml.	R.B.C. count per ml.	Highest dilution enhancing growth (twofold) at 24 hr*: Virulent 544	Virulent 2308	Attenuated 45/0	Attenuated 58/20
8×10^8	$1·7 \times 10^9$	1/60	1/60	1/60	1/60
5×10^8	$2·6 \times 10^9$	1/60	1/60	1/60	1/60
5×10^8	$2·7 \times 10^8$	—	1/60	1/30	1/30
6×10^8	—		1/60	1/30	1/30
Four red blood cell diffusates		All inactive 1/10 for 2308			
Four plasma diffusates		All inactive 1/10 for 2308			

* Growth measured by viable counts; medium tryptic digest meat broth (1 vol.) and isotonic saline (2 vol.); inoculum 100 organisms/ml.

strains. The factor(s) capable of stimulating both virulent and avirulent strains of *B. abortus* was apparently confined to leucocytes as all erythrocyte and plasma fractions were inactive.

DISCUSSION

The finding that erythritol stimulates virulent more than attenuated strains of *Brucella abortus* is interesting in view of the previous investigations (Williams *et al.* 1962) suggesting that the presence of foetal erythritol is a cause of the localization of *B. abortus* in bovine foetal tissue. However, the lack of erythritol in bovine white blood cells suggests that this differential effect of erythritol on virulent *B. abortus* strains probably has no relevance to the primary invasive process of intraphagocytic survival and growth. In contrast to these results with *B. abortus* there seems to be no correlation between growth stimulation by erythritol and the virulence of various strains of *B. melitensis* and of *B. suis*.

The fact that extracts of the mixed cell population of bovine 'buffy coat' stimulate the growth of *Brucella abortus* is interesting. If the same growth-stimulating activity were found in extracts of phagocytes purified from the buffy coat, it might have some relevance to the intracellular growth of virulent *B. abortus*.

A factor that may be involved in the phenomenon of intracellular survival and growth in bovine phagocytes is glutamic acid metabolism. Olsen (1951) showed that there was a correlation between virulence and high oxidative rate of glutamic acid in *Brucella abortus* and we have shown the influence of L-glutamic acid on the growth of avirulent strains in a synthetic medium. Furthermore, Anderson & Smith (1965) have shown that stimulation of growth of a virulent strain by erythritol is inhibited by high concentrations of glutamic acid. Wilson & Dasinger (1960) have discussed the influence of glutamic acid in relation to intracellular growth. They suggest that the rapid removal of intracellular glutamate by avirulent strains might produce conditions inimical to the organisms' growth.

We are indebted to Dr W. J. Brinley Morgan, Professor J. B. Wilson and Dr J. H. Payne for providing strains and to Miss G. R. Fanstone, Mrs P. Stenhouse, Miss A. D. Rawkins and Mr G. Wilkins for excellent technical assistance. Statistical analysis was done by our colleague Mr S. Peto. Part of this work was used by one of us (A.E.W.) in a thesis submitted for the degree of Doctor of Philosophy in the University of London.

REFERENCES

ANDERSON, J. D. & SMITH, H. (1965). The metabolism of erythritol by a virulent strain of *Brucella abortus*. *J. gen. Microbiol.* **38**, 1.

COPELAND, J. M. (1955). *Characterization of a stimulating factor for* Brucella abortus *from yeast autolysate.* M.S. Thesis Univ. Wis. Madison, Wis.

KEPPIE, J., WILLIAMS, A. E., WITT, K. & SMITH, H. (1965). The role of erythritol in the tissue localization of the brucellae. *Brit. J. exp. Path.* (in the Press).

MACRAE, R. M. & SMITH, H. (1964). The chemical basis of the virulence of *Brucella abortus*. VI. Studies on immunity and intracellular growth. *Brit. J. exp. Path.* Vol. 45.

McCULLOUGH, N. B. & BEAL, G. A. (1951). Growth and manometer studies of carbohydrate utilization by Brucella. *J. infect. Dis.* **89**, 266.

OLSEN, C. B. (1951). *The aerobic metabolism of* Brucella abortus. M.S. Thesis Univ. Wis. Madison, Wis.

Pearce, J. H., Williams, A. E., Harris-Smith, P. W., Fitzgeorge, R. B. & Smith, H. (1962). The chemical basis of the virulence of *Br. abortus*. II. Erythritol, a constituent of bovine foetal fluids which stimulate the growth of *Br. abortus* in bovine phagocytes. *Brit. J. exp. Path.* **43**, 31.

Rode, L. J., Oglesby, G. & Schuhardt, V. T. (1950). Cultivation of Brucella on chemically defined media. *J. Bact.* **60**, 661.

Smith, H. & Fitzgeorge, R. B. (1964). The chemical basis of the virulence of *Brucella abortus*. V. The basis of intracellular survival and growth in bovine phagocytes. *Brit. J. exp. Path.* **45**, 174.

Smith, H., Keppie, J., Cocking, E. C. & Witt, K. (1960). The chemical basis of the virulence of *Pasteurella pestis*. I. The isolation and the aggressive properties of *Past. pestis* and its products from infected guinea pigs. *Brit. J. exp. Path.* **41**, 452.

Smith, H., Williams, A. E., Pearce, J. H., Keppie, J., Harris-Smith, P. W., Fitzgeorge, R. B. & Witt, K. (1962). Foetal erythritol: a cause of the localization of *Brucella abortus* in bovine contagious abortion. *Nature, Lond.* **193**, 47.

Williams, A. E., Keppie, J. & Smith, H. (1962). The chemical basis of the virulence of *Brucella abortus*. III. Foetal erythritol, a cause of the localization of *Brucella abortus* in pregnant cows. *Brit. J. exp. Path.* **43**, 530.

Wilson, J. B. & Dasinger, B. L. (1960). Biochemical properties of virulent and avirulent strains of Brucellae. *Ann. N.Y. Acad. Sci.* **88**, Art. 5, 1155.

J. gen. Microbiol. (1964), 37, 293–295
Printed in Great Britain

Adenine Inhibition of the Rate of Sorocarp Formation in *Dictyostelium discoideum*

By M. I. KRICHEVSKY and L. L. LOVE

U.S. Department of Health, Education, and Welfare, Public Health Service, National Institutes of Health, National Institute of Dental Research, Bethesda, Maryland 20014, *U.S.A.*

(*Received* 29 *July* 1964)

SUMMARY

Some characteristics of adenine inhibition of the rate of sorocarp formation by *Dictyostelium discoideum* are described. As little as $1{\cdot}25 \times 10^{-3}$ M-adenine was markedly inhibitory. All of the stimulants tested (histidine, glucose, Mg^{2+}, K^{+}, PO_4^{3-}, Na^{+}) were capable of stimulating the rate of fructification even in the presence of adenine, although to a lesser extent. It is concluded that the inhibition by adenine and the stimulation by the other materials listed are independent phenomena.

INTRODUCTION

The ability of a variety of materials to stimulate the rate of development in *Dictyostelium discoideum* was described previously (Krichevsky & Wright, 1963; Krichevsky & Love, 1964). In connexion with this work it was decided to investigate the phenomenon of adenine inhibition of morphogenesis as reported by Bradley, Sussman & Ennis (1956). The present report further characterizes the inhibition of development by adenine.

METHODS

The procedures for growing and harvesting the myxamoebae, as well as for determining the effect of materials on the rate of morphogenesis, were as described previously (Krichevsky & Wright, 1963). The usual method was to incubate all test plates and a control plate containing plain 2·5 % agar until the control plate was about to finish forming its first complete sorocarp. If one waits long enough, all plates would contain approximately equal numbers of mature sorocarps. Thus, the number of sorocarps at the time of counting is a measure of the stimulation of the relative rate of sorocarp formation in the presence of the test substance as compared with the water control. However, in determining adenine inhibition, the control plate containing only water was allowed to achieve almost complete fructification before comparative counts were made.

RESULTS

As reported by Bradley *et al.* (1956) adenine is capable of inhibiting the rate of aggregation when compared to water controls. When the criterion of measurement of the inhibitory capacity of adenine was that of comparative rate of sorocarp formation, the results shown in Fig. 1 were obtained. The degree of inhibition was approximately proportional to adenine concentration at the lower concentrations.

The effect of adenine on the rate of sorocarp formation in the presence of 0·02 M-

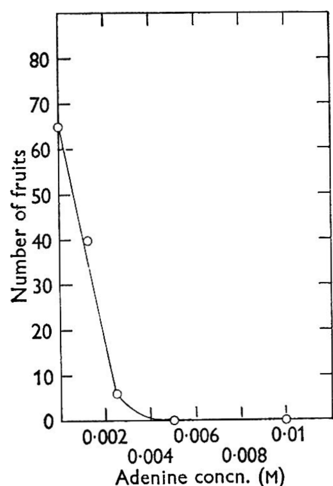


Fig. 1

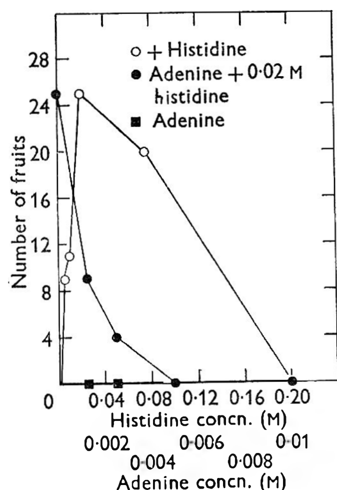


Fig. 2

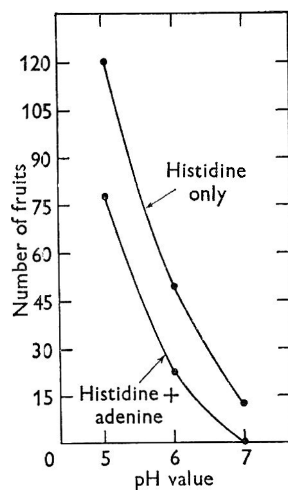


Fig. 3

Fig. 1. Inhibition of sorocarp of Dictyostelium discoideum formation by adenine. The medium was adjusted to pH 6·2 in all cases.

Fig. 2. The effect of adenine on sorocarp formation of Dictyostelium discoideum in the presence of L-histidine. All samples were at pH 6·2. The L-histidine final concentration was 0·02 M when used with the indicated concentrations of adenine. Three 0·01 ml. drops of amoebal suspension were added per test Petri dish instead of the usual six drops.

Fig. 3. The effect of histidine + adenine at various pH values on sorocarp formation by Dictyostelium discoideum. The final adenine concentration was 4×10^{-3} M; that of the L-histidine was 4×10^{-2} M.

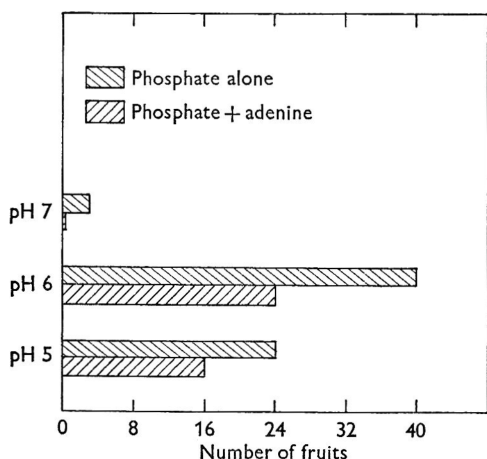


Fig. 4

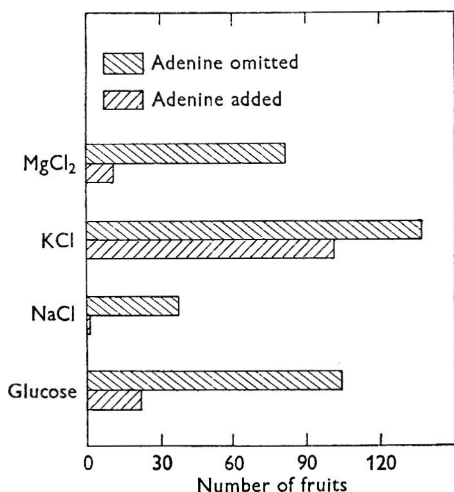


Fig. 5

Fig. 4. The effect of phosphate and adenine combinations at various pH values on sorocarp formation by Dictyostelium diseoideum. The final concentration of adenine was 4×10^{-3} M. Sodium phosphate buffers were added to a final phosphate concentration of 10^{-2} M, at the pH values indicated.

Fig. 5. Sorocarp formation by Dictyostelium discoideum in the presence of adenine and various stimulants. Where indicated, additions were made to final concentrations as follows: Adenine, 4×10^{-3} M; glucose, 0·05 M; NaCl, 0·03 M; KCl, 0·03 M; $MgCl_2$, 0·015 M.

histidine (pH 6·2) is illustrated in Fig. 2. It may be seen that the variation in the rate of sorocarp formation with concentration of adenine was approximately the same regardless of the presence or absence of histidine (compare with Fig. 1). The concentration curve for histidine alone is presented for comparison.

It was found (Fig. 3) that, in the presence of 0·04 M-histidine, adenine had approximately the same relative inhibitory effect regardless of the pH value of the medium. As described previously (Krichevsky & Wright, 1963), histidine stimulated in inverse relationship to the pH value. In contrast, no relationship was observed which could be ascribed to the pH value of the adenine solution. This last observation is further illustrated (Fig. 4) by the effect of sodium phosphate buffer and adenine. The difference in sorocarp formation between those samples containing adenine and those without was about the same at pH 5 and pH 6. Since sodium phosphate was only slightly stimulatory at pH 7 (in contrast to the same concentrations of potassium phosphate at pH 7 as reported previously; Krichevsky & Wright, 1963) it was not possible to make the same test at this pH value.

The effect of adenine with other stimulants of morphogenesis is shown in Fig. 5. Those compounds which best stimulated the rate of fructification (i.e. glucose, KCl, $MgCl_2$) were able to do so even in the presence of adenine. In contrast, NaCl, which was a poor stimulant alone (Krichevsky & Wright, 1963) did not stimulate to any discernible extent in combination with adenine.

DISCUSSION

In the main, materials which had been found to stimulate sorocarp formation in *Dictyostelium discoideum* did so most efficiently at concentrations greater than 0·1 M. In contrast, adenine was markedly inhibitory at much lower concentrations. None of the materials tested in presence of adenine appeared capable of modifying the effect of adenine. That is, the rate of sorocarp formation was less in the presence of adenine than without adenine. This result was qualitatively similar, irrespective of the other components present in the test system. Since no interaction was noted between adenine inhibition of the rate of sorocarp formation and stimulation of it by those materials tested (with the possible exception of K^+), it would appear that the mechanisms by which the inhibition and stimulation are effected differ from one another. That is, both adenine inhibition and metabolite stimulation are observed simultaneously. It was suggested previously (Krichevsky & Love, 1964) that histidine stimulated by changing the permeability of the amoebae. It has been observed that histidine and other stimulants do indeed modify the exit of some macromolecular materials (namely ribonucleic acid, protein) from cells (Krichevsky & Love, unpublished data). Thus it seems unlikely that the mechanism of adenine inhibition involves changing the permeability of the developing amoebae.

REFERENCES

BRADLEY, S. G., SUSSMAN, M. & ENNIS, H. L. (1956). Environmental factors affecting the aggregation of the cellular slime mold, *Dictyostelium discoideum*. *J. Protozool.* **3**, 33.

KRICHEVSKY, M. I. & LOVE, L. L. (1964). The uptake and utilization of histidine by washed amoebae in the course of development in *Dictyostelium discoideum*. *J. gen. Microbiol.* **34**, 483.

KRICHEVSKY, M. I. & WRIGHT, B. E. (1963). Environmental control of the course of development in *Dictyostelium discoideum*. *J. gen. Microbiol.* **32**, 195.

[*The Editors of the* Journal of General Microbiology *accept no responsibility for the Reports of the Proceedings of the Society. Abstracts of papers are published as received from authors.*]

THE SOCIETY FOR GENERAL MICROBIOLOGY

The Society for General Microbiology held its forty-first General Meeting at the Queen's University of Belfast, on Thursday, Friday and Saturday, 17, 18 *and* 19 *September* 1964. *The following communications were made:*

ORIGINAL PAPERS

Presumptive Evidence for a Regulatory System Controlling Capsular Synthesis in *Bacillus anthracis*. By G. G. MEYNELL (*Guinness-Lister Research Unit, Lister Institute of Preventive Medicine, London*) and ELINOR MEYNELL (*Department of Bacteriology, Wright-Fleming Institute of Microbiology, London*)

The capsular material of the anthrax bacillus consists largely, if not entirely, of polymerized D-glutamic acid (PGA). Capsule formation by wild-type strains requires incubation in air + CO_2 to produce a threshold concentration of bicarbonate ion (HCO_3^-) in the medium (Meynell, E. & Meynell, G. G. (1964), *J. gen. Microbiol.* **34**, 153). HCO_3^- therefore acts as an inducer of PGA synthesis. Mutant strains are known which form PGA in air without added CO_2. These may therefore be constitutive for PGA synthesis and arise from mutation in a gene controlling the PGA biosynthetic pathway. This view is supported by the chemical identity of the PGA formed by wild-type and mutant strains, as well as by other characteristics to be described.

PGA synthesis by the wild-type is inhibited by fatty acids which, we have suggested, may act by interfering with CO_2 fixation. Consistent with this is the finding that the mutants which do not require additional CO_2 for PGA synthesis are capsulated on nutrient agar; whereas the wild-type is not, unless adsorbents like serum or charcoal are added to inactivate naturally-occurring fatty acid in the medium. Moreover, comparison of cultures incubated in CO_2 shows that capsule formation by mutants is far less readily inhibited by known fatty acids or by lipid extracted from agar.

Phage Patterns of Rough Mutants of *Salmonella typhimurium* strain LT2. By R. G. WILKINSON and B. A. D. STOCKER (*Guinness-Lister Research Unit, Lister Institute of Preventive Medicine, London*)

In *Salmonella* spp. mutations causing roughness, probably through inability to make (or to attach) the smooth-specific side-chain of the normal somatic lipopolysaccharide, alter phage sensitivity. Phages P22(C_2), P22h(C_2) and Felix O, and the 'rough-specific' phages P221(C_1). 6SR, Ffm, 60 and C21 were tested on mutants of *S. typhimurium* LT2, selected either for roughness, defects in carbohydrate metabolism, or resistance to phage P22.

All of 6 *rouB* rough mutants, which cannot manufacture the rhamnose-containing smooth side-chain (Subbaiah, T. V. *et al.* (1964), *Nature, Lond.* **201**, 1298), were sensitive to Felix O, 6SR, P221, Ffm and 60 ('rough-sensitive' pattern). Some *rouA* mutants, which synthesize smooth-specific side-chains but do not attach them, had the same pattern, but most differed by resistance to 6SR and Felix O ('rough-resistant' pattern). Many further mutants with either 'rough-sensitive' or 'rough-resistant' phage-phenotypes have not been investigated genetically or biochemically.

Mutants deficient in UDP-galactose-epimerase (and so unable either to ferment galactose or to synthesize UDP-galactose from glucose) were resistant to P22 (Fukasawa, T. & Nikaido, H. (1960), *Virology*, **11**, 508) and P22h, sensitive to the 'rough-specific' phages Ffm, 60 and P221, and also to C21, which in LT2 as in *E. coli* K12 attacks mutants unable to incorporate galactose into the cell wall (Shedlovsky, R. & Brenner, S. (1963), *Proc. nat. Acad. Sci., Wash.* **50**, 300); when grown with galactose (and glucose, to relieve their galactose-sensitivity), these LT2 mutants become sensitive to P22 (Fukasawa, T. & Nikaido, H., *loc. cit.*), P22h and Felix O and resistant to the 'rough-specific' phages,

including C21. Galactose non-fermenting mutants deficient only in galactokinase were of smooth (wild-type) phage sensitivity. A mutant of strain LT7 lacking all three enzymes of the galactose pathway had the 'galactose-epimerase' phage pattern, even when grown with galactose. Most mutants with this phage pattern were galactose non-fermenting, presumably either 'galactose-epimeraseless' or 'triple' mutants; several, which ferment galactose, are perhaps unable to transfer it from UDP-galaetose to the lipopolysaccharide.

We received from Dr M. J. Osborn and S. Rosen four mutants blocked between mannose-6-phosphate and fructose-6-phosphate, a conversion involved both in mannose fermentation and in synthesis of CDP-mannose from glucose. All had the characteristic phage pattern of sensitivity to 60, 6SR, P221, P22h and Felix O. Growth with mannose restored the smooth (wild-type) phage phenotype. Rough mutants of several less common phage patterns await genetical and biochemical investigation.

An Effect of Ethionamide on *Mycobacterium tuberculosis*: Fall in Carbohydrate Content. By F. WINDER and P. BRENNAN (*Department of Biochemistry and Laboratories of the Medical Research Council of Ireland, Trinity College, Dublin*)

Exposure of growing *Mycobacterium tuberculosis* to isoniazid results in an early fall in its content of certain lipid and bound carbohydrate fractions, and an accumulation of certain other lipids and of soluble sugar, which is mainly trehalose (Winder, F. (1964), *Biochim. biophys. Acta*, 82, 210; Winder, F. & Brennan, P. (1964), *Biochim. biophys. Acta*, **90**, 442; also unpublished observations). As part of a programme to determine whether this pattern is specific to isoniazid, the effect of α-ethyl-thioisonicotinamide (ethionamide; Rist, N. (1960), *Adv. Tuberc. Res.* **10**, 69) on this organism was studied.

Ethionamide (100 μg./ml.) was added to young cultures of BCG growing in modified Sauton medium on a rotary shaker. These and control cultures were harvested and fractionated according to the above references. Twelve hours' exposure to ethionamide reduced soluble sugar from 1·1 to 0·7, carbohydrate in the lipid fraction from 1·0 to 0·4 and other carbohydrate from 3·0 to 2·0 (all calculated as mmoles of glucose equivalent per g. of protein). There was no detectable effect on the content of RNA or DNA per g. of protein, and little effect on total lipid, during this period.

These results are different from those obtained with isoniazid. They suggest that ethionamide has a specific effect on carbohydrate synthesis.

Role of Extraneous Hydrogen Acceptors for *Leuconostoc* in Glucose Fermentation. By J. MEYRATH (*University of Strathclyde, Glasgow*)

Members of the genus *Leuconostoc* isolated from Swiss wines and fruit juices grew considerably faster and gave a higher yield of cells with arabinose or fructose as sources of carbon than with glucose. Stimulatory effects of pentoses have been known to occur in heterofermentative lactic acid bacteria, but no explanation regarding the mechanism has been given. Although the mentioned property makes our strains differ markedly from the three type species of *Leuconostoc*, this seems not sufficient justification for assigning the organisms to a separate species. Moreover, there is evidence that the fermentation mechanism of hexoses and pentoses by our strains correspond qualitatively to those characteristic for *Leuconostoc* and established by De Moss, Bard & Gunsalus ((1951), *J. Bact.* 62, 499) and other workers.

Our strains show a stronger production of acetic acid per cell in glucose and arabinose substrates than do other *Leuconostoc* spp., and the ratio of ethanol to acetic acid is lower for our strains than for others. There seems to be a certain inability in our strains to reduce acetic acid to ethanol which is an essential step in glucose fermentation by *Leuconostoc* and not necessary in pentose fermentation; this appears to be at least one reason for the slower growth on glucose than on arabinose. This explanation is supported by the fact that glucose is a very good source of carbon for our strains if pyruvic, oxalacetic, or citric acid is added. These acids seem to function as better hydrogen acceptors than the intermediate acetic acid. Other strains of *Leuconostoc* have been shown to be stimulated by acetic acid (De Moss *et al. loc. cit*) and for these the rate-limiting step in glucose fermentation was

thought to be the formation of the intermediate hydrogen acceptor acetic acid, rather than its ability to be reduced to ethanol.

Photoinactivation of Vaccinia Virus. By G. S. TURNER (*Lister Institute of Preventive Medicine, Elstree, Hertfordshire*)

Photodynamic inactivation was first applied to vaccinia virus by Perdrau, J. R. & Todd, C. ((1933), *Proc. Roy. Soc.* B, **112**, 288) and Herzberg, K. ((1933), *Z. ImmunForsch.* **80**, 507). Photoinactivation re-examined by modern techniques of virus assay had parameters similar to those observed with other viruses. Using methylene blue (MB) as the photosensitizer, exponential survival curves indicated a 'single hit' reaction (Lea, D. E. (1955), *Actions of Radiations on Living Cells.* 2nd edn. London: C.U.P.) like those found with ultraviolet and ionizing radiations. Such effects proceeded to completion, were irreversible, independent of temperature and of the intensity of illumination which affected rate only. Inactivation was dependent on the pH and on the presence of oxygen. Optimal MB concentration was $1-3 \times 10^{-5}$M and higher concentrations did not increase inactivation; active wavelengths were those maximally absorbed by MB (630–730 mμ). Photoinactivation appeared to require dye-virus combination. Ion-exchange resins detached dye before and after inactivation; thus dye-virus combination is probably ionic and reversibly dissociable. Increase of photoinactivation with increasing pH suggested greater dye binding at higher pH.

Photoinactivation was inhibited by highly polymerized ribose and deoxyribose nucleic acid solutions but not by individual bases or pentoses. Inhibition by nucleic acid cannot be explained by changes produced in MB absorption spectra but probably by competition for MB. Only relatively high concentrations of extraneous protein partially inhibited photoinactivation. Some viscous solutions including inert polymers inhibited photoinactivation but whether this inhibition was due to chemical properties or to poor diffusion of oxygen is uncertain.

Photoinactivation is known to involve oxidation but was unaffected by highly active preparations of catalase and peroxidase. Whether the site of action is virus protein or nucleic acid is being investigated. Photoinactivation as an effective method for producing immunogenic inactivated vaccine is reported by Kaplan, C. ((1964), *Proc. Soc. gen. Microbiol.* abstract below).

Immunogenicity of Non-Infectious Smallpox Vaccines. By C. KAPLAN (*Lister Institute of Preventive Medicine, Elstree, Hertfordshire*)

Two sorts of non-infectious smallpox vaccine were used. Both were prepared from partially purified suspensions of vaccinia virus grown in the skins of sheep. The first was inactivated by ultraviolet irradiation (Collier, L. H., McClean, D. & Vallet, L. (1955), *J. Hyg., Camb.* **53**, 513). This vaccine, after successful tests in rabbits, was injected intramuscularly into ten previously vaccinated subjects; all had increases in neutralizing antibody—some very large (Kaplan, C. (1962), *Lancet*, ii, 1027). Twenty 1-year-old children were then immunized with two doses of the vaccine. Serum samples were taken before each injection and 3 weeks after the second. Most of the children had neutralizing antibody after one dose; all had after two. They were challenged by live virus vaccination immediately after the third bleed. All but two developed typical primary vaccinia. It is uncertain how this is related to immunity to either vaccinia or smallpox.

Exposure of vaccinia virus to ultraviolet must be carefully controlled. Over exposure destroys immunogenicity as well as infectivity. Inactivation of vaccinia virus infectivity by photodynamic action of dyes is less critical. Advantage was taken of this to prepare the second vaccine. Tests to date have been confined to animals. The immunogenicity of vaccine inactivated by long periods of illumination is not impaired; the resistance of rabbits immunized by such vaccines to challenge with potent virus was the same as in animals immunized with vaccine illuminated for the minimum lethal time (Turner, G. S. (1964), *Proc. Soc. gen. Microbiol.* abstract above); the antibody levels in the two groups

of rabbits were also comparable. Delayed hypersensitivity may be a concomitant of immunization with non-infectious smallpox vaccine as it is of conventional vaccination. The relationship between delayed hypersensitivity and immunity to smallpox remains to be determined.

Immunity Produced by Formalin-killed Vaccinia Virus Vaccine. By B. J. W. BEUNDERS (*Inspectie van de Militair Geneeskundige Dienst, Naussauplein 30, Holland*)

The study of immunity following small-pox vaccination has one advantage over studies on immunity following vaccination with other viral vaccines. The grade of immunity can be indicated by observing the skin reaction following scarification with live calf-lymph vaccine. In no other human virus vaccination can immunity be tested by direct observations of skin reactions to challenge with live virus; antibody estimations are then the only method for providing information on immunity. Unfortunately, although the titre of neutralizing antibody provides some estimate of the efficiency of a given vaccine, it is not always a reliable criterion of immunity.

The results of a 6-year study with a formalin-killed vaccinia virus vaccine have led to the following schedule of immunization. Four intradermal injections (0·2 ml.) of the inactivated vaccine are given and the procedure is repeated 6 weeks and 6 months later. Estimations of the level of neutralizing antibody 2 weeks after the last injection are made.

The Immunization of Rabbits with Inactivated Vaccinia Virus. By E. A. BOULTER, H. T. ZWARTOUW and D. H. TITMUSS (*Microbiological Research Establishment, Porton, Wiltshire*)

During an investigation unrelated to the study of inactivated virus vaccines it was noted that a single injection of killed, purified vaccinia virus with adjuvant elicited extremely high levels of neutralizing antibody in rabbits. The potential importance of this observation for the prophylaxis of human smallpox stimulated a study of the factors critical to the immunogenic potency of killed vaccinia virus. The results indicate that two key factors are the amount of antigen injected and the use of an adjuvant. Maximum titres of antibody, often exceeding 10^6, were obtained with 1·0 mg. of antigen suspended in Freund's complete adjuvant. With mineral oil alone used as adjuvant, as in human influenza vaccines, titres of about 50,000 were obtained, while aluminium hydroxide gave slightly lower titres; in the latter case a fairly rapid fall in titre was evident within 6 weeks. The potentiating effect of oily adjuvants was due, at least in part, to a prolongation of the antibody response. Preliminary experiments suggest that about 0·04 mg. of virus, corresponding to approximately 4×10^8 pock-forming units, is needed to produce antibody titres in excess of 10^4. There was little difference between formalin-inactivated and ultraviolet-irradiated preparations. Immunized rabbits were completely resistant to respiratory challenge with virulent rabbit pox virus.

The Immunizing Effect of Purified Measles Virus Haemagglutinin. By E. C. J. NORRBY, R. LAGERCRANTZ and S. GARD. (*Department of Virus Research, Karolinska Institutet, School of Medicine and the Pediatric Clinic, Karolinska Sjukhuset, Stockholm, Sweden*)

Measles virus material harvested from primary dog kidney cultures was treated with Tween 80 and ether after which the haemagglutinin (HA) of the virus was isolated in a purified state by physico-chemical methods. The product, which retained the virus-specific immunogenic activity, was suspended in phosphate-buffered saline and used as a vaccine (TE vaccine) in studies which also included a formalin killed, adjuvant vaccine (FK vaccine) prepared in monkey kidney tissue cultures. Although comparative potency tests in guinea pigs indicated that the TE vaccine was 3 to 4 times more potent than the FK vaccine, almost identical results were obtained after immunization (three monthly doses) of children, aged ½–2 years. Both vaccines produced 100% conversions and the geometrical means of the serum titres were about 1:180 and 1:600 in neutralization and HI

tests, respectively. Low titre HI antibodies were still detectable in the sera from all immunized children 1 year after vaccination. Two mild cases of measles occurred during this period among children given the FK vaccine, but none among those given the TE vaccine.

A comparative study was also made of the effect of one injection of either of the two vaccines given to children immunized 18 months previously with three monthly doses of FK vaccine. The increase in geometrical means of the serum titres were 31- and 102-fold with the FK and TE vaccine respectively. The mean of the serum titres in the latter group of children was about 7 times higher than the mean titre reached in early measles convalescent sera; 1:18,900 as compared to 1:2560.

Immunization of Baboons against Trachoma and Inclusion Conjunctivitis. By L. H. Collier, W. Blyth (*M.R.C. Trachoma Research Unit, Lister Institute of Preventive Medicine, London*), J. Treharne and N. M. Larin (*Biologicals Research Department, Pfizer, Ltd., Sandwich, Kent*)

Inoculation of the baboon conjunctiva with some strains of trachoma/inclusion conjunctivitis (TRIC) agents induces characteristic follicular conjunctivitis and formation of epithelial cell inclusion bodies. By scoring the features of this syndrome numerically, a quantitative measure of the severity of infection can be obtained. This system was used to compare the responses to conjunctival challenge of immunized and control animals.

For most experiments, inclusion conjunctivitis strain MRC-4/ON was used as challenge since it induces a moderately severe and easily recognizable infection. Live preparations made from the homologous strain grown in chick embryos or HeLa cells induced virtually complete immunity when given as two subcutaneous doses and one intravenous dose at weekly intervals; although waning, immunity was sometimes detectable more than a year later. Replacement of the final intravenous dose by a subcutaneous injection conferred partial protection only; no better results were obtained by using a mineral oil adjuvant.

Formolized vaccines appeared to confer some immunity, but the results were variable; and inactivation at 37° or by ultraviolet irradiation completely destroyed immunogenicity.

Both live and formolized vaccines induced formation of complement-fixing antibody, but titres diminished rapidly during the 3 months after immunization; the relation of this antibody to the state of immunity is not clear.

The finding that some inadequately immunized animals reacted more severely than controls to conjunctival challenge suggests the possibility of sensitization.

Antigens prepared from other TRIC agents were either ineffective or only partially effective against challenge with MRC-4/ON; and some of these agents failed even to protect against challenge with the homologous strain.

It is hoped that these tests will provide a useful guide to the efficacy of vaccines designed to control trachoma in man, but this will not be known until the results can be compared with those of field trials.

Repeating Structure in Influenza Virus Filaments. By T. H. Flewett (*Regional Virus Laboratory, Birmingham*)

Straight filaments of influenza A_2 and C viruses concentrated by centrifugation were examined by the electron microscope using the negative-staining technique. Composite prints were made in which the negative image was moved along the paper at various intervals. This revealed a hexagonal pattern. Possible fallacies arising in the interpretation of superimposed micrographs of this kind are discussed.

Influence of a Non-ionic Surface Active Agent on Coliphage Infections. By B. W. Janicki, M. J. Rose, Jr. and S. A. Aron (*Veterans Administration Hospital, Washington, D.C., U.S.A.*)

Earlier studies of the effect of non-ionic surface active agents on bacteriophage infections of *Escherichia coli* were designed primarily to determine the effect of treating the virus with

detergents before infection of untreated host bacteria. Adsorption to the host was not affected by exposing T1 and T2 phages to Tween 80 (Groman, N. B. & Bobb, D. (1955), *Virology*, **1**, 313) and no effect on the ability of gamma coliphage to infect its host was found on pre-incubation in Triton NE (Klein, M., Kalter, S. S. & Mudd S. (1945), *J. Immunol.* **51**, 389). Our studies were conducted to determine the effect of cultivation of *E. coli* in Triton WR-1339 (polyoxyethylene ether of octyl phenol) on infection by untreated phages of the T2 serotype.

Studies of growth cycles of the T2, T4, and T6 coliphages demonstrated that the adsorptive phase and the length of the latent period were unaffected by cultivation of host bacteria in nutrient broth containing 0·5% Triton. However, the burst size of treated cultures was significantly less than that of control cultures. Phage particles pretreated with Triton were not inactivated neither were growth rates and the characteristic physiological reactions of the host bacteria affected by cultivation in Triton. The phage growth cycle data demonstrated that Triton did not inhibit bursting of infected bacteria. Triton also did not appear to alter bacterial fragility since no difference in susceptibility of treated and untreated cultures to disruption by sonic vibration was noted. These observations suggest that the reduced yield of phage from treated cultures may result from an alteration of some metabolic pathway essential to phage replication. Preliminary studies support this possibility; the glucose dehydrogenase activity of Triton cultures was found to be significantly less than that of control cultures.

Further Experiments on Rhinovirus Vaccines. By JENNIFER E. DOGGETT, P. J. CHAPPLE, D. A. J. TYRRELL and M. L. BYNOE (*Common Cold Research Unit, Salisbury*)

Inactivated and live rhinovirus vaccines combined with Arlacel-Drakeol adjuvant were administered to volunteers. Antibody responses occurred but these were no greater than those produced by the same vaccines without adjuvants.

Under suitable conditions rhinoviruses can be adsorbed to aluminium phosphate and can be eluted from it. Virus adsorbed on to aluminium phosphate induced antibody responses in man and rabbits.

Antibody produced in volunteers with live or inactivated vaccine was found to persist for at least 12–18 months, and to protect against infection with homologous virus.

Parainfluenza Virus Type 2 and Type 3 Vaccines in Infants and Adult Volunteers. By H. W. KIM, R. H. PARROTT, J. O. ARROBIO, J. C. CANCHOLA and A. J. VARGOSKO (*Children's Hospital of the District of Columbia, Washington, D.C. U.S.A.*)

Alum and Drakeol-Arlacel forms of inactivated vaccines to parainfluenza types 2 and 3 were tested for their antigenicity, safety and effectiveness in adults and infants. No immediate or delayed untoward reactions have occurred after intramuscular inoculation. In adults with no prior detectable antibody, there was a 100% conversion rate 190–200 days following inoculation with alum adsorbed and Drakeol-Arlacel emulsified vaccines to parainfluenza type 2. Seventy per cent of infants with no prior antibody exhibited a significant antibody rise 130–160 days after first inoculation with the alum adsorbed parainfluenza type 2 vaccine; only 10% responded to Drakeol-Arlacel parainfluenza type 2 vaccine.

Thirty per cent of adults, all of whom had prior antibody, showed a four-fold or greater antibody rise to three inactivated parainfluenza type 3 vaccines.

The Response to Challenge with Type 1 Poliovirus of Children Immunized with Inactivated or Attenuated Poliovaccines. By J. L. HENRY, E. S. JAIKARAN (*London County Council*), JOAN R. DAVIES, A. J. H. TOMLINSON (*Public Health Laboratory Service, County Hall, London*), P. J. MASON, JOAN M. BARNES and A. J. BEALE (*Glaxo Research Ltd., Sefton Park, Stoke Poges, Bucks*)

Four groups, each containing about 50 children, were recruited at age 2 months at Infant Welfare Centres. Group A were given three doses of quadruple vaccine (diphtheria,

tetanus, whooping cough and poliomyelitis) at age 2, 3 and 4 months. Group B were given triple antigen (i.e. without the poliomyelitis component) at the same ages and acted as a group of unimmunized controls. Group C were given the same primary course as Group A and received a booster dose of quadruple vaccine at age 15 months. Group D were given the same primary course as Group B followed by trivalent Sabin vaccine at 6, 7 and 8 months.

Groups A and B were challenged with graded doses of Sabin type 1 vaccine virus at age 6 months and Groups C and D at 16 months. Specimens of faeces were examined at monthly intervals from the time of recruitment at age 2 months until challenge, in order to detect natural poliovirus infection. After challenge the duration and amount of virus excreted were determined. Children were bled for antibody studies at appropriate times.

The unimmunized controls, after challenge, excreted large amounts of virus for at least 3 weeks. The children immunized with inactivated poliovaccine, Groups A and C, excreted rather less virus for shorter periods. The children in Group D required a larger dose of challenge virus to infect them and, if infected, excreted only small amounts of virus.

Replication of Riley's Plasma Enzyme Elevating Virus *in vitro*. By R. Evans (*Cancer Research Department, London Hospital Medical College, London*)

Replication of Riley's Virus (RV) in mouse embryo tissue cultures was first reported by Yaffe (1962, *Cancer Res.* **22**, 573). He maintained the virus indefinitely by serial weekly passage of supernatant medium to fresh primary cultures. RV could not be maintained by subculturing infected cells or in secondary cultures. The loss of RV from cultures after 2–3 weeks has been reported (Adams, D. H., Rowson, K. E. K. & Salaman, M. H. (1961), *Brit. J. Cancer*, **15**, 860; Georgii, A. & Lenz, I. (1964), *Nature, Lond.* **202**, 1228), but the replication of RV in tissue cultures has not been studied in any detail. Cultures were therefore infected with virus, unabsorbed virus washed off after 1 hr and the infectious virus titre in the supernatant followed. The titre commenced to rise after 7 hr, reached a peak after 16–24 hr, and slowly fell to zero in 8–10 days. Success in obtaining virus replication depended on the length of time the cells had been in culture before infection. Cultures inoculated when 7 days old yielded lower virus titres and became sterile more rapidly than cultures inoculated 24 hr after trypsinization, and 10–14-day-old cultures did not support virus replication. Replicate cultures were assayed for free and total virus at intervals. Intracellular virus did not rise appreciably before the extracellular virus and always formed a low proportion of the total virus. No difference was observed between RV growth curves in cultures prepared from whole mouse embryo, embryo liver, adult spleen or peritoneal macrophages. No replication occurred in HeLa cells or Rhesus monkey kidney cells. The failure of virus to replicate in older cultures could be due to a physiological change in the cells or to the death of a susceptible cell type.

SYMPOSIUM: 'INACTIVATED VIRUS VACCINES'

Inactivated Poliovirus Vaccine. By A. J. Beale (*Glaxo Research Ltd. Stoke Poges, Bucks*)

During the 10 years since inactivated tissue culture poliovaccines were first used, much has been learned about the factors involved in successful application of inactivated virus vaccines. First, it was established that there are three serological types of poliovirus and that specific neutralizing antibody will passively protect animals and man against the paralytic consequences of poliovirus infection. Also, it is now clear that many other enteroviruses can cause paralytic poliomyelitis, although fortunately they rarely do so. Secondly, methods for large-scale production of high titre poliovirus free from nervous tissue were developed after the demonstration by Enders, Weller and Robbins that poliovirus would grow in primate cells in culture. These methods have been extensively studied, and the yield of poliovirus has been greatly increased in terms of concentration and amount of virus from a monkey. Thirdly, a method was discovered for inactivating virus with formaldehyde. The conditions required to inactivate all the poliovirus demand filtration of virus before formalin treatment. Extensive tests for residual virus have to be

carried out on large volumes of vaccine. It soon became apparent that these tests for residual virus had to be prolonged, because partially inactivated virus takes much longer than untreated virus to produce cytopathic effects in cell cultures. Extraneous viruses derived from monkey kidneys soon began to cause trouble in the safety tests. Fortunately, most of these simian viruses are readily inactivated by formalin, but one (SV 40) produced tumours in hamsters and transformation of human cells in culture; and it is resistant to formalin inactivation. It can be excluded from vaccine, or it can be inactivated by the photodynamic action of certain dyes, for example, toluidine blue. The presence of extraneous viruses, difficult to inactivate, is a powerful argument for abandoning primary monkey kidney cells for vaccine production although such inactivated poliovaccine has in practice proved safe since the Cutter disaster.

The effectiveness of inactivated vaccine was first shown by the field trial conducted by Francis. Although effective, early vaccines were deficient in potency of both type 1 and type 3 components. This deficiency was particularly serious when immunization was attempted in young infants possessing maternally transmitted antibodies. It has been found possible, by increasing the antigenic mass of inactivated poliovaccines, to produce satisfactory antibody to all three components. The responses of older individuals are better than those of younger infants.

In controlling the potency of inactivated vaccine, an important step has been identification and measurement of the antigen responsible for eliciting neutralizing antibodies. This is the D antigen. Although inactivated vaccines can produce amounts of antibodies as high as those achieved by natural or deliberate poliovirus infection, the duration and quality of the immunity has remained in doubt. The evidence at present suggests that the rate of decline of antibody titre is the same whether produced by inactivated or natural infection. The antibodies that appear in the blood of an individual vaccinated with inactivated poliovaccine of high potency are 7S in type, associated with long-lasting immunity.

Inactivated poliovaccine has had a profound effect on the epidemiology of poliomyelitis and must have limited spread of the virus. Studies of individuals who have had inactivated vaccine and have been fed attenuated virus, have shown little increase in the dose of living virus required to infect, compared with unvaccinated subjects, but the virus excreted by these vaccinated individuals is less both in duration and titre.

Measles Vaccines. BY P. B. STONES (*Pfizer Ltd., Sandwich, Kent*).

A measles vaccine first became a practical possibility with the isolation of virus strains in monkey kidney cells (Enders, J. F. & Peebles, T. C. (1954), *Proc. Soc. exp. Biol., N.Y.* **86**, 277). These workers selected one strain ('Edmonston') for further study and after serial passage in human kidney and amnion cultures, the virus was adapted to chick embryo tissue. Chick adapted virus was shown to have considerably reduced pathogenicity in monkey and man, but still retained a sufficient degree of virulence to cause frequent febrile and exanthematous reactions. Unsuccessful attempts to overcome these by different routes of administration are described. Further passage (Schwarz, A. J. F. (1962), *Amer. J. Dis. Child.* **103**, 386) has resulted in a decrease, but not complete removal, of these side effects. The simultaneous administration of live vaccine and gamma globulin has been widely practised in the United States with reduction in incidence of reactions, but possibly a less effective immunity.

Killed measles vaccines have also been the subject of much study, and while it is clear that a satisfactory immunity can be obtained with three doses of an alum-precipitated vaccine, it is of relatively short duration. Killed vaccine of good potency presents a good substitute for gamma globulin in eliminating side-effects of live vaccine, one dose of killed being given approximately one month before the live vaccine.

The standardization of live vaccine can be achieved in terms of its infectivity for tissue culture and the potency expressed in terms of TCID50. It is valuable in this context to specify the type of tissue used, as considerable variation in sensitivity occurs. An alternative is the establishment of a reference material of accepted titre.

Standardization of killed vaccine must be correlated with field efficacy and this necessitates the use of a reference vaccine which has undergone trials in children. The development and technique of an extinction limit potency test is described.

The future of measles vaccination and consideration of different vaccination policies and schedules are discussed.

Inactivated Vaccinia Virus Vaccines. By T. A. McNeill (*Department of Microbiology, The Queen's University of Belfast*)

Jennerian vaccination as prophylactic for smallpox is undoubtedly very effective. However, as a result of its often unpleasant, and sometimes serious side effects, many investigations have been made into the immunogenicity of inactivated vaccinia virus preparations with the ultimate object of developing an inactivated vaccine which would be as effective as the living vaccine but which would be free from complications.

Another approach to the problem of reducing vaccination reactions has been to use an inactivated vaccinia virus vaccine to precede vaccination with live virus, so that the vaccinee is provided with a basal immunity which protects him against the vaccination illness without interfering with the effectiveness of the vaccination. Herrlich and his associates in Germany, and Verlinde and his associates in the Netherlands have been most active in studying this aspect, and their trials in humans have given promising results.

The fundamental problem underlying inactive vaccinia virus vaccines is to define the conditions under which the inactive virus might be immunogenic. Thus, an outstanding feature of the literature on this subject is the divergence of results between those investigators who have found inactive virus immunogenic, and those who have found it either very poorly immunogenic or else completely ineffective. This problem is not made any easier by the great diversity of tests for, and criteria of immunogenicity used by, different investigators.

In order to provide a more composite picture of the problem, a study has been made of the immunogenicity of inactivated vaccinia virus taking the following factors into consideration: type of inactivant, extent of inactivation, route of inoculation and dose. Vaccines were prepared with highly purified virus and immunogenicity for rabbits was measured by (i) development of neutralizing antibody, and (ii) development of skin resistance to graded doses of live virus. The evidence suggests that the dose of inactive virus is the most important single factor in producing a good immune response. It seems probable that with larger doses of antigen than have been used in most of the previous studies, better protection could be produced.

One disturbing feature is that some of the rabbits in the experiments mentioned above developed severe hypersensitivity reactions in the skin at the sites of challenge with live virus.

If a potent inactivated vaccine can be produced, further problems arise. If such a vaccine is to replace Jennerian vaccination it must obviously be shown to be as effective in preventing smallpox. A pre-requisite for this is more basic research into the factors influencing immunity to smallpox in order that a preliminary laboratory assessment of such a vaccine could be made. It would obviously be difficult to justify the trial of a completely unknown vaccine in the face of a smallpox outbreak when we already have a well tried vaccine available.

If pre-treatment with inactivated vaccinia virus before live virus vaccination is to become more generally acceptable, immunization schedules will have to be carefully worked out. The effect of pre-treatment on subsequent immune response to live vaccine, and the problem of inducing hypersensitivity reactions of a severe type requires further investigation.

Inactivated Influenza Virus Vaccines. By D. Hobson (*Evans, Medical Ltd., Liverpool*)

There is no satisfactory experimental model of influenza, hence vaccines can be evaluated only by extensive field trials. Provided attention is focused on serological and epidemiological results obtained with virus strains strictly homologous with those in the vaccine, significant antibody induction and clinical protection can always be demonstrated

(Meiklejohn, G., Kempe, C. H., Thelman, W. G. & Lennette, E. H. (1952), *Amer. J. Hyg.* **55**, 12). The antigen responsible for inducing protection is the viral haemagglutinin (HA), and haemagglutinin-inhibiting (HI) antibody levels can usually be correlated with the degree of clinical protection obtained (Salk, J. E., Bailey, M. L. & Laurent, A. M. (1945), *Amer. J. Hyg.* **42**, 57; Meiklejohn *et al.* (1952); Seal, J. R. & Gundelfinger, B. F. (1958), *U.S. Armed Forces Med. J.* **9**, 1720). However, the major HA component is highly specific for each family of strains, and, especially in Type A viruses, these antigens have shown considerable and progressive variation over the last 30 years. The precise nature of this antigenic shift is still disputed (Jensen, K. E. & Francis, T. Jr. (1953), *J. exp. Med.* **98**, 619; Andrewes, C. H. (1950), *New Engl. J. Med.* **242**, 197) but the change is often of sufficient magnitude to alter the whole epidemic pattern of the disease, with the new variant quickly replacing all the foregoing epidemic strains. When this occurs, all population-immunity induced by earlier strains becomes irrelevant to the current situation. Thus vaccines of influenza A strains failed to protect against A^1 epidemics (Meiklejohn *et al.* 1952) and A plus A^1 vaccines were equally valueless against the Asian influenza epidemic (Medical Research Council Report (1958), *Brit. Med. J.* i, 414).

The one clear-cut requirement for influenza vaccine is that the newly emerging epidemic strain must be included without delay. The resulting difficulties in availability and potency of a new antigen from poorly laboratory-adapted strains, required to act as a primary immune stimulus in an inexperienced population, have repeatedly resulted in poor antibody induction, confined to 70 % or less of the population, with a rapid decline of antibody levels (Salk, J. E. (1953), *J. Amer. med. Ass.* **151**, 1169; Davenport, F. M., Hennessy, A. V., Hauser, H. B. & Cryns, W. F. (1956), *Amer. J. Hyg.* **64**, 304) and of clinical protection (Meichen, F. W., Rogan, E. & Howell, R. W. (1962), *Brit. J. indust. Med.* **19**, 203). The antibody induced tends to be narrow and confined to the vaccine strain (Salk (1953); Hobson, D. & Pearson, E. (1961), *Brit. J. exp. Path.* **42**, 53) and protection may not even extend to further minor variants of the same virus family (Langmuir, A. D., Henderson, D. A. & Serfling, R. E. (1964), *Amer. J. publ. Hlth*, **54**, 563).

Attempts to improve the immunogenicity of vaccine by increasing the antigenic mass or the number of doses are limited by the availability of the new vaccine strain by the toxicity of the virus itself (Kato, N. & Hara, H. (1960), *Brit. J. exp. Path.* **42**, 145) and the accompanying foreign host proteins. In general, the effect of such manipulations has been slight (Salk *et al.* (1945)) and attention has turned to the augmentation of vaccine potency by immunological adjuvants based on the work of Freund, J. (1951), *Amer. J. clin. Path.* **21**, 645). Emulsifications of vaccine in mineral oil alone increased the height and duration of antibody response in a higher proportion of people than saline vaccine (Salk, 1953; Davenport, F. M. (1961); *J. Allergy*, **32**, 177) and the breadth of response was greater. The amount of antigen required for effective immunization could be reduced considerably in the emulsified product. The clinical effectiveness of oil adjuvant vaccines has been confirmed with a variety of epidemic strains (Davenport *et al.* 1956; Meiklejohn, G. (1962), *J. Amer. med. Ass.* **179**, 594).

The next essential step if immunological adjuvants are to be used rationally is to define and purify the influenza antigens required to induce protection. Fairbrother, R. W. & Hoyle, L. (1937), *J. Path. Bact.* **44**, 213) showed that viral HA could be separated by ether extraction and by precise extensions of this method (Davenport, F. M., Rott, R. & Schafer, W. (1959), *Fed. Proc.* **18**, 563) purified and potent HA components of Asian and pre-Asian influenza strains have been obtained and used in vaccine trials (Davenport, F. M., Hennessy, A. V., Brandon, F. M., Webster, R. G., Barrett, C. D. & Lease, G. O. (1964), *J. lab. clin. Med.* **63**, 5) with successful immunological results and a significant reduction in the toxic effects often associated with influenza vaccination.

Inactivated Vaccines in Foot-and-Mouth Disease. By J. B. BROOKSBY *(The Animal Virus Research Institute, Pirbright, Surrey)*

Production

The first widely used foot-and-mouth disease vaccines (Waldmann, O. & Kôbe, K. (1938), *Berl. tierärztl. Wschr.* 3 June, 317) were prepared from virus harvested from the tongues of

infected cattle. This technique produced satisfactory vaccines but the disposal of infected carcasses raised serious problems. Within 10 years, Frenkel (Frenkel, H. (1947), *Bull. Off. int. Epiz.* **28**, 155) had developed a method for the production of virus in the surviving cells in epithelial slices collected from the tongues of cattle slaughtered for meat. Although virus titres in such cultures rarely equalled those of 'natural' virus, it was shown (Henderson, W. M. & Galloway, I. A. (1953), *J. Hyg., Camb.* **61**, 337) that, virus for virus, comparable vaccines were of equal potency. Frenkel's technique requires fairly sophisticated apparatus and a good supply of tongue tissue. The demonstration of susceptibility of various kidney cells in tissue culture provided an alternative source of virus and this has been exploited on a large scale (Ubertini, B., Nardelli, L., Panina, G., Santero, G., Michelsen, E. & Morgen, H. C. (1961), *Bull. Off. int. Epiz.* **55**, 1433) using primary culture of calf kidney. A further advance to the use of cell lines is in progress.

From all these sources of virus, vaccines have most frequently been prepared by the method of Waldmann and Köbe, i.e. inactivation by 1/2000 formalin with aluminium hydroxide at 25° and pH 9·0. Inactivation by formalin in the absence of aluminium hydroxide is slow; Brown *et al.* (Brown, F., Hyslop, N. St G., Crick, Joan & Morrow, A. W. (1963), *J. Hyg., Camb.* **61**, 337) have demonstrated live virus after 144 hr incubation. Combination of formalin and adsorbent gives a safe product. For inactivation β-propiolactone has been shown by Fellowes *et al.* (Fellowes, O. N., Edward, A. G., Tessler, J., Poppensiek, G. C. & Sharp, J. B. (1959), *Amer. J. vet. Res.* **20**, 992) to be satisfactory and acetylethyleneimine by Brown *et al.* (1963) but, although inactivation may be more regular, the resultant vaccines have no greater protective power than the formalin-aluminium hydroxide type.

Control

Assuming standard conditions for inactivation, a correlation might be expected to exist between the highest virus titre reached during growth in the starting material and immunogenic potency of the vaccine; and Henderson & Galloway (1953, *J. Hyg., Camb.* **51**, 546) found that for the strains studied by them about $10^{6 \cdot 8}$ ID50 were necessary to provide one PD50 for cattle.

A more direct method, as yet inapplicable to the standard vaccine containing aluminium hydroxide, is measurement of the complement-fixing activity of the inactivated material after treatment with Arcton. This activity is directly related to the concentration of 25 mμ inactivated particles which constitute the immunogenic material.

Tests for non-infectivity of foot-and-mouth disease vaccines have been dealt with at length by Henderson (1952, *J. Hyg., Camb.* **50**, 195). The chance of failing to detect small amounts of infectivity must be appreciated. Even if 100 observations are made and all are negative, on statistical grounds 3·0 % of samples could be infective. Routine practice has been to inoculate 0·1 ml. of undiluted vaccine at each of 20 sites in the tongues of 6 cattle.

Potency tests on the finished product may be by inoculation of cattle, followed either by challenge or by antibody assay, or (less satisfactorily) by inoculation of other species, usually guinea pigs or mice, again with challenge or antibody assay.

Field application

Foot-and-mouth disease vaccines must meet the local requirements in respect of antigenic relation to virus or subtype. This complexity is one of the greatest obstacles in immunization against the disease, with seven types and an ever-increasing number of subtypes (Davie, J. (1963), *Proc. 17th World Vet. Congr. Hannover*, 675). Good vaccines challenged with homologous virus will regularly give 100 % protection at 21 days after inoculation but immunity wanes gradually through the ensuing 6 months. Revaccination gives a good secondary response and is an important factor in building up an immune population (van Bekkum (1960), *Bull. Off. int. Epiz.* **54**, 372).

Inactivated Newcastle Disease Vaccine. By S. A. Keeble (*Glaxo Laboratories Ltd., Greenford, Middlesex*)

Inactivated vaccines have for the past 2 years been used in England and Wales to control Newcastle Disease of poultry, previously controlled by slaughter of affected birds. Vaccine was therefore used initially in a totally susceptible population.

Both β-propiolactone and formaldehyde have been used in the preparation of commercial vaccine. The source of virus is either allantoic fluid from infected eggs or fluid from tissue culture preparations. Either virulent or attenuated strains of virus can be used apparently with equal success. Response to vaccination can be assessed by measuring the development of serum-neutralizing (S.N.) antibodies or of serum-haemagglutination inhibition (H.I.) or by protection against challenge. There is a direct relation between the antigen content of a vaccine and the amounts of S.N. and H.I. antibodies formed. A similar relationship can be shown between vaccine dosage and challenge in vaccinated birds.

Safety and potency standards specified by the Ministry of Agriculture determine freedom from live virus, as detected by egg and chicken inoculation, and adequate immunogenic activity, as determined by direct challenge of vaccinated birds. This is one of the few virus vaccines for which direct challenge can be used in potency tests. This is possible because (*a*) the small size and cost of chickens enables relatively large numbers to be used; (*b*) the pathogen is virulent, killing 100 % of controls in 6 days; and (*c*) the vaccine is potent and rapidly gives strong protection. In the tests, 0·5 ml. of a 1:50 dilution of vaccine must protect 50 % of 5-week-old birds when challenged intramuscularly with 200,000 EID50 of virulent virus 14 days after vaccination.

An adjuvant is incorporated in the vaccine. Usually this is 20–40 % v/v aluminium hydroxide gel, but oil adjuvants have been used. Aluminium hydroxide leaves no lesion at the site of inoculation.

When injected intramuscularly into 21-day-old chicks, 0·5 ml. vaccine will protect for more than 12 weeks, which is adequate for broilers killed at 10 weeks old. If the interval between first and second doses is adequate, the second dose will give an anamnestic response in which H.I. and S.N. titres rise considerably. Laying birds are usually vaccinated at 3 weeks and again at 16–20 weeks, with an additional dose at 9 weeks if the flock is in an area of high disease incidence. This regime should give protection throughout the life of the bird, i.e. until 18 months of age.

Maternal antibodies pass to progeny via the yolk, and at 4 days of age the antibody level may be as high as in the hen, though the antibodies usually disappear by 21–28 days of age. Though these antibodies protect individual chicks against experimental challenge and cause serious interference with the action of the vaccine, they give no substantial flock protection and are therefore a liability rather than an asset.

The uptake of vaccine by the poultry industry has been less than expected, but the number of outbreaks reported in the 12 months since the end of the slaughter policy is 2760, compared with an average for the previous 4 years of 2379, indicating a similar degree of control to that given by the slaughter policy, but at a fraction of the cost. In a survey of 235 flocks, which did not include broiler flocks, the protection rates for once, twice, three and four times vaccinated birds were 56 %, 87 %, 95 % and 98 %, respectively.

Egg production is a sensitive indicator of flock health and is depressed by Newcastle disease. In a survey of 187 flocks, egg production fell to nil in 81 % of unvaccinated flocks and was normal in 1 %. In vaccinated flocks, egg production in only 18 % fell to nil, and in 20 % there was no fall in production. The reduction lasted 12 days in vaccinated and 17 days in unvaccinated birds.