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The Nucleic Acid Contents of Viruses

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SUMMARY

The nucleic acid contents of purified suspensions of mouse pneumonitis organisms (Nigg), pox viruses, adenovirus type 5, the iridescent virus of Tipula and avian myeloblastosis virus were analysed. From these results and published information the absolute amounts of nucleic acid in each virus particle were calculated and are presented in tabular form. Some generalizations can be made about the nucleic acid composition of different groups of organisms reproduced in cells; these are discussed in relation to the possible evolutionary origins of the organisms.

INTRODUCTION

Knight (1954) pointed out that although plant viruses differ considerably in size they all have nearly the same absolute content of ribonucleic acid (RNA). Frisch-Niggemeyer (1956) calculated that the same amount of RNA, representing a molecular weight of about 2 millions, is also present in animal viruses (influenza, fowl plague, equine encephalitis and poliomyelitis). In the present paper it will be shown that the generalization about the absolute nucleic acid content can be extended to include the cytoplasmic RNA viruses of insects. The absolute nucleic acid contents of other viruses and organisms multiplying within cells have been calculated from published information and the results of our own analyses of several preparations.

From this compilation some other generalizations emerge. In the first place, organisms belonging to the rickettsia and psittacosis-lymphogranuloma groups contain both deoxyribonucleic acid (DNA) and RNA, even in highly purified preparations. Both kinds of nucleic acid seem to be essential constituents of these organisms; loss of RNA is associated with a decrease in infectivity. This fact, together with several others discussed below, suggests that organisms in the rickettsia and psittacosis-lymphogranuloma groups have closer affinities with bacteria than with any of the true viruses, the genetic apparatus of which probably consists of either DNA or RNA, but not both (Lwoff, 1957). Secondly, it seems that the absolute nucleic acid contents of most DNA viruses, whether they infect bacteria, insect or vertebrate cells, fall within a relatively limited range, corresponding to molecular weights of 60–150 millions—that is, from 30 to 75 times as much nucleic acid as in the RNA viruses. Two groups of viruses are exceptional in having much lower DNA contents. These are the small, spherical bacteriophages, one member of which is known to have a single-stranded DNA, and certain mammalian tumour viruses. Our own observations were made in an attempt to fill in some of the more

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important gaps in available information on the nucleic acid contents of viruses and other organisms reproduced within cells. In the discussion these results are tabulated along with published information on other viruses.

METHODS

Highly concentrated and purified suspensions of mouse pneumonitis organisms, pox viruses, adenovirus type 5, the iridescent virus of Tipula and avian myeloblastosis virus were analysed by the diphenylamine technique of Burton (1956) for DNA and the method of Webb (1956) for RNA. Protein was determined by the technique of Lowry, Rosebrough, Farr & Randall (1951), with bovine albumin as a standard. Methods of preparation of suspensions are described along with results.

In most of the preparations analysed, electron microscopic spray counts of particles were made by Dr R. C. Valentine as described by Williams & Backus (1949). Counts of larger organisms were made in smears delivered from loops of known volume (Hart & Rees, 1960) calibrated with ^{131}I-labelled albumin, and stained by the acridine orange technique (Armstrong & Niven, 1957). In the discussion, where particle counts were not available, absolute nucleic acid contents were calculated from the % nucleic acid, the size and the density of the particles. The results are expressed as molecular weights of nucleic acid per particle, since this figure allows a direct comparison between different viruses and provides an estimate of the amount in each one.

Assessment of the purity of virus suspensions is always difficult. We took as criteria of purity: (1) absence of significant contamination visible on electron microscopy and acridine orange staining; (2) comparison of the amount of nucleic acid recovered in the suspension with that expected of the corresponding number of virus particles, their size, density and % nucleic acid being known. In the case of vaccinia virus an additional check on the purification procedure was used. To crude unlabelled virus preparations homogenates of cells labelled with ^{32}P were added. The radioactivity of the purified virus preparation could be taken as a measure of host cell contamination, and proved to be less than 20%.

RESULTS

Mouse pneumonitis organism (Nigg). The Hampstead strain of this organism was grown in mouse embryo cell cultures prepared as described by Allison & Armstrong (1960). Cells were homogenized in a Potter-type apparatus and the material submitted to differential centrifugation followed by chromatography on modified cellulose columns (Hoyer *et al.* 1958). The suspensions of organisms obtained showed, when stained with acridine orange, the bright yellow-green fluorescence characteristic of DNA. Both DNA and RNA were found on analysis (Table 2). The value for RNA may be low because of loss during preparation.

Pox viruses. The Lister Institute rabbit-adapted strain of vaccinia virus (Amies, 1938), in its 81st passage in rabbit skin, was used. The cowpox virus was also a Lister Institute strain, distinguishable from vaccinia virus in that only cowpox gave haemorrhagic lesions on the chorioallantoic membrane of chick embryo. Material grown in rabbit skin was purified by three cycles of extraction with trifluorotrichloroethane (Arcton 113) (Gessler, Bender & Parkinson, 1956), differential centrifugation and repeated flocculation with M-sodium chloride

(Dawson & McFarlane, 1948). Virus was dispersed in distilled water by brief exposure to ultrasonic vibrations from a magnetostriction transducer.

Electron micrographic spray counts of the final preparations gave particle counts of the order of 2 to 3×10^{11}/ml. All preparations gave concentrations of DNA of 2·46 to $2{\cdot}92 \times 10^{-16}$ g./particle. Two preparations of vaccinia virus and one of cowpox virus contained small but definitely detectable amounts of RNA. This RNA was probably due to residual contamination with host material, since after further salt flocculation and dispersion only a trace of colour was given by the *p*-bromophenylhydrazine reagent. If the colour was due to RNA, then the amount of RNA was less than one-thirtieth of the amount of DNA in the preparations. However, the absorption maximum at 450 mμ characteristic of *p*-bromophenylhydrazone (Webb, 1956) was absent, suggesting that the trace of colour was probably due to some non-specific reaction, possibly with carbohydrate. We therefore conclude that pox virus particles contain about $2{\cdot}6 \times 10^{-16}$ g. DNA/particle and no RNA. The particles showed bright yellow-green fluorescence when stained with acridine orange.

Adenovirus. Three highly concentrated preparations of adenovirus type 5, grown in HeLa cells and prepared as described by Pereira & Valentine (1958), were available for study. Centrifugation in a caesium chloride density gradient (carried out in collaboration with Dr H. G. Pereira) showed that the particles had a density of 1·34. All three preparations showed the presence of DNA in amounts estimated at 1·2 to $2{\cdot}3 \times 10^{-16}$ g./particle. Two of the preparations showed traces of RNA, the third no detectable RNA. The latter also had the lowest DNA content, and was thought to be without significant host cell contamination. It is therefore tentatively concluded that adenovirus type 5 has about $1{\cdot}2 \times 10^{-16}$ g. DNA/particle, the nucleic acid content being about 30%; and no RNA. This result is consistent with the density of the particle.

The iridescent virus of Tipula. A suspension of this virus was purified by Arcton extraction and differential centrifugation. Electron micrographic spray counts showed the presence of $2{\cdot}7 \times 10^{11}$ particles/ml. The amount of DNA recovered corresponded to $2{\cdot}3 \times 10^{-16}$ g./particle, representing a nucleic content of 16%; no RNA was detectable.

Avian myeloblastosis virus. This virus was prepared and purified as described by Bonar & Beard (1959). The RNA content of the virus was 1%, with good agreement in duplicates; no DNA was detected.

DISCUSSION

Information about the absolute nucleic acid contents of viruses and other organisms reproduced intracellularly is summarized in Tables 1–4. In Table 1, two organisms, *Escherichia coli* and *Mycoplasma gallisepticum*, both of which multiply in the absence of cells, are included for comparison. Certain generalizations about the nucleic acid contents of these organisms can now be made with some confidence.

Rickettsias

Smith & Stoker (1951) reported that *Rickettsia burneti* grown in yolk sacs of chick embryos contained 9·7% (dry weight) of DNA and 4·3% of RNA (Table 2). Different preparations were found to have the same % DNA but variable concentrations of RNA, which was nevertheless consistently present. The purine and pyrimidine

Table 1. *DNA and RNA contents of various organisms*

Group	Organism	Shape	Dimensions (mμ)	DNA %	DNA mol. wt. (× 10^{-6})	RNA %	RNA mol. wt. (× 10^{-6})
Gram-negative bacteria	*Escherichia coli*	Rod	3000 × 1000	4·4 (1)	25,000	22·6	130,000
Pleuropneumonia-like organism	*Mycoplasma gallisepticum*	Spherical	250 diameter	6 (2)	75	?	?
Rickettsia	*R. burneti*	Rod	600 × 250	9·7 (3, 4)	1680	4·3	749
	R. mooseri	Rod	600 × 300	0·9 (5, 6)	224	3·0	747
Psittacosis-lymphogranuloma	Meningo-pneumonitis	Spherical	270 diameter	5–10 (7)	1200	0 (?)	0 (?)
	Feline pneumonitis	Spherical	270 diameter	6 (8)	1400	16	3740
	Mouse pneumonitis	Spherical	270 diameter	6·5 (P)	1520	4·7	1190

References are given in parentheses. P—personal observations (see text). (1) Roberts *et al.* (1955); (2) Morawitz & Cleverden (1959); (3) Smith & Stoker (1951); (4) Cohen & Chargaff (1944); (5) Wyatt & Cohen (1952); (6) Cohn *et al.* (1958); (7) Crocker (1952); (8) Ross & Gogolak (1957).

base composition of the rickettsial DNA was found to be similar to that of chick embryo, except that chick embryo contained 5-methylcytosine. To account for the similarity in base composition, the authors mention the possibility that rickettsias may incorporate nucleic acid directly from the host. However, Wyatt & Cohen (1952) found a very different base composition in the DNA of *Rickettsia prowazeki* grown in chick embryo (Table 2). This large difference between the two organisms, if substantiated, resembles the considerable differences in DNA base ratios among bacteria, to which attention has been drawn (Belozersky & Spirin, 1958; Marmur & Doty, 1958) and which raises some interesting problems in connexion with the coding of genetic information. In both rickettsias the adenine : thymine and guanine : cytosine ratios were close to unity, which suggests strongly that the DNA exists in the double-stranded helical form with base-pairing as described by Watson & Crick (1953).

Table 2. *DNA bases in rickettsias* (% *proportional*)

Organism	Adenine	Thymine	Guanine	Cytosine	Reference
R. burneti	29·5	26·0	22·5	22·0	(1)
R. prowazeki	35·7	31·8	17·1	15·4	(2)

(1) Smith & Stoker (1951); (2) Wyatt & Cohen (1952).

Cohn, Hahn, Cegalowski & Bozeman (1958) reported that both DNA and RNA were present in purified *Rickettsia mooseri*. Assuming a nitrogen content of 13 % (Smith & Stoker, 1951; Cohen & Chargaff, 1944), their figures correspond to 0·8 % DNA and 2·9 % RNA. This figure for DNA is considerably less than that given for *R. burneti* by Smith & Stoker, and it remains to be seen whether the discrepancy is genuine. Cohn *et al.* (1958) found that on incubation of suspensions of *R. mooseri* at 36° RNA was liberated into the medium. There was a concomitant decrease in the infectivity of the preparations which was not prevented by nucleotide supplements. Lability of RNA could account also for the low and variable recoveries of RNA in *R. burneti* by Smith & Stoker.

Psittacosis-lymphogranuloma group

Histochemical studies suggested that the feline pneumonitis organism contains both DNA and RNA (Moulder & Weiss, 1951). The first physicochemical analysis of purified meningopneumonitis organisms, however, gave particle weights of 2·5 to 5×10^{-14} g. with 5–10 % DNA and no demonstrable RNA (Crocker, 1952). In calculating the absolute amounts of nucleic acid in organisms belonging to this group, we have assumed that they are spherical, with diameters of 270 mμ, as found in freeze-dried preparations (Crocker & Williams, 1955). Much greater diameters are observed in air-dried specimens examined by electron microscopy.

Zahler & Moulder (1953) reported that purified feline pneumonitis organisms have both DNA and RNA. From the given values of DNA- and RNA-phosphorus and total nitrogen/LD_{50} dose, it can be calculated that the organisms had approximately 6 % DNA and 16 % RNA. Ross & Gogolak (1957) reported results of nucleic acid base analyses of purified psittacosis and feline pneumonitis organisms which indicated the presence in both of DNA and RNA. Our results show that the mouse pneumonitis organism also has both RNA and DNA. Although only DNA fluorescence is visible when suspensions of particles are examined after acridine orange staining, substantial amounts of RNA are apparent when the organisms are developing in mouse embryo cells, as shown by Pollard, Starr, Moore & Tanami (1960) for psittacosis grown in human amnion cells.

In general, it appears to be established that all organisms in the rickettsia and psittacosis-lymphogranuloma groups contain both DNA and RNA. The variable recoveries of RNA, and the fact that in one analysis of the meningopneumonitis organism RNA was not found, are probably due to the relative lability of RNA, which can diffuse out of the particles easily, whereas DNA is more firmly bound. Loss of RNA may well be one of the factors responsible for the loss of infectivity of these organisms on incubation at 36°, even in the presence of nucleotide supplements.

In addition to the consistent presence of both nucleic acids in these organisms, several findings imply closer relationship with bacteria than with the true viruses. Rickettsias undergo binary fission (Schaechter, Bozeman & Smadel, 1957). The sensitivity of members of the rickettsia and psittacosis-lymphogranuloma groups to sulphonamides and tetracyclines, and the failure of any true viruses to be affected by these drugs, have been known for some time (Werner, 1958). Recently, it has been shown that rickettsias and members of the psittacosis-lymphogranuloma groups have cell walls similar to those of bacteria, consisting of mucopeptides containing muramic acid and digested by lysozyme (Allison & Perkins, 1960). The sensitivity of these organisms to penicillin in relatively high dosage may be due to inhibition of cell-wall synthesis; this is the main effect of penicillin so far known in bacteria (Park, 1958; Rogers & Perkins, 1959). Pox viruses and myxoviruses do not possess cell walls of this kind, and penicillin even in very high doses is without effect on virus growth.

All these findings suggest that organisms of the rickettsia and psittacosis-lymphogranuloma groups are probably bacteria which have become adapted to intracellular multiplication. Perhaps they have lost the capacity to synthesize certain enzymes necessary for growth and replication, and so have become obligate

intracellular parasites. In the rickettsias of insects there seems to be no sharp line of distinction between intracellular and free-living forms (Steinhaus, 1946). Moreover, typical bacteria are known (e.g. *Mycobacterium lepraemurium*) which multiply only within animal cells.

Organisms of the rickettsia and psittacosis-lymphogranuloma groups have somewhat more DNA than the large DNA viruses or *Mycoplasma gallisepticum*, which is a free-living pleuropneumonia-like organism (Morawitz & Cleverden, 1959).

RNA viruses

As previously pointed out (Knight, 1954; Frisch-Niggemeyer, 1956; Schäfer, 1959), the RNA contents of plant and animal viruses for which published figures are available are of the same order, representing molecular weights of about 2 millions. The data listed in Table 3 show that this generalization can be extended to include the only two insect cytoplasmic viruses so far analysed, so far as we are aware.

Table 3. *Nucleic acid contents of RNA viruses*

Group	Virus	Shape	Dimensions or diameter (mμ)	RNA %	RNA mol. wt. ($\times 10^{-6}$)	References
Rod-like plant viruses	Tobacco mosaic	Rod	300 × 15	5·7	2·2	(1, 2)
	Tobacco rattle	Rod	180 × 25	5·0	2·7	(2, 3)
	Potato X	Rod	600 × 10	5·7	2·6	(2)
Spherical plant viruses	Turnip yellow mosaic	Sphere	22	35	2·0	(2, 4)
	Southern bean mosaic	Sphere	25	20	1·9	(2, 5)
	Tomato bushy stunt	Sphere	30	17	2·1	(2, 6)
	Tobacco necrosis	Sphere	23	18	1·5	(2, 7)
Spherical vertebrate virus	Poliomyelitis	Sphere	27	25	1·75	(8)
	Foot and mouth	Sphere	25	40	3·1	(9, 10)
	Encephalomyocarditis	Sphere	27	30	2·10	(11)
Arborvirus	Equine encephalitis	Sphere	50	4·4	2·2	(8)
Myxovirus	Influenza	Sphere	80	0·8	2·3	(8)
	Fowl plague	Sphere	70	1·8	2·7	(8)
Avian tumour	Myeloblastosis	Sphere	80–110	2·3	4·9	Present study
Insect cytoplasmic	From *Dasychira pudibunda*	Sphere	40	7	2·5	(12, 13)
	From *Sphinx populi*	Sphere	80	0·9	2·6	(12)

(1) Hopkins & Sinsheimer (1959); (2) Paul (1959); (3) Harrison & Nixon (1959); (4) Markham & Smith (1951); (5) Miller & Price (1946); (6) de Fremery & Knight (1955); (7) Bawden & Pirie (1945); (8) Schäfer (1959); (9) Ströhmaier & Mussgay (1959); (10) Bradish *et al.* (1960); (11) Faulkner *et al.* (1960); (12) Bergold (1958); (13) Krieg (1956).

Certain points deserve comment. The figure for the total amount of nucleic acid in foot-and-mouth disease virus is based on sedimentation analysis of extracted RNA (Ströhmaier & Mussgay, 1959). If this figure and the recent observations suggesting particle diameters of 24–25 mμ (Bradish, Henderson & Kirkham, 1960) are correct, then the virus has an unusually high % RNA. The RNA content of tobacco rattle virus given in Table 3 is based on the large particle size observed (Harrison & Nixon, 1959) since the smaller particles are not infective, although they have the same density as the larger particles. There is some uncertainty about the dimensions

of tobacco necrosis virus, and the rather low figure for total RNA calculated for this virus may have to be revised.

The figure given for the avian myeloblastosis virus is based on our own analysis, which gave similar proportions of RNA in the virus to the published figure of 2·2 % (Bonar & Beard, 1959).

DNA viruses

Bacteriophages. The DNA contents of several bacteriophages have been published. The T2, T4 and T6 coliphages have about the same size and phosphorus content, representing a total DNA content of about 2×10^{-16} g. and a molecular weight of DNA of about 120 millions/organism (Table 4). The smaller T1, T3, T5 and T7 coliphages have somewhat lower phosphorus contents, which are of the same order in all. Taking Lunan & Sinsheimer's (1956) figure for T7 as representative, 61 % of the total P is present in DNA, and each particle has $5{\cdot}8 \times 10^{-17}$ g. DNA, giving a molecular weight of 35 millions. Phosphorus analyses suggest that the DNA contents of Salmonella P22 and Pyocyanea P2 and P8 temperate phages are

Table 4. *Nucleic acid contents of DNA viruses*

Group	Virus	Shape	Dimensions (mμ)	DNA %	DNA mol. wt. × 10^{-6}	References
Large bacteriophage	T2, T4, T6	Prismatic + tail	95 × 65 (head)	40	120	(1, 2)
	T1, T3, T7		50 × 45 (head)	40	35	(3, 4)
			45 × 45 (head)	45	60	(4)
	Salmonella P22		50 × 45 (head)	40	35	(4)
	Pyocyanea P2, 8	?	?	?	50	(4)
Small bacteriophage	X174	Spherical	37 diameter	25	1·7	(5)
Insect nuclear	From *Bombyx mori*	Rod	279 × 40	13	76	(6)
	From *Lymantria dispar*		300 × 30	16	56	(7)
	From *Aporia crataegi*		220 × 50	9	58	(8)
Insect cytoplasmic	Tipula iridescent	Icosahedral	130 diameter	16	156	Present study
Pox virus	Vaccinia	Brick	260 × 220 × 220	7·8	156	Present study
	Cowpox			7·2	156	Present study
Adenovirus	Adenovirus type	Icosahedral	70 diameter	30	66	Present study
Animal tumour	Rabbit papilloma		45 diameter	8	7	(9)

(1) Cohen (1957); (2) Stent & Fuerst (1955); (3) Lunan & Sinsheimer (1956); (4) Stent (1958); (5) Sinsheimer (1959); (6) Bergold (1947); (7) Smith & Wyatt (1951); (8) Bergold (1958); (9) Watson & Littlefield (1960).

slightly greater than that of T7 (Stent, 1958), and DNA contents have been calculated on the assumption that in these phages also 61 % of the phosphorus is in DNA. In contrast to these results, Sinsheimer (1959) found that the small phage ϕX174 had a very low DNA content, corresponding to a molecular weight of 1·7 millions. The DNA extracted from these organisms had the same molecular weight, from which it seems that all the DNA in the phage particle is present in a single molecule. Evidence was presented that the polynucleotide chain was single stranded, in

contrast to the usual double-stranded form. Interest has been aroused by the recent description by Loeb & Zinder (1961) of an RNA-containing bacteriophage.

Pox viruses. Very little has been published on the DNA content of mammalian viruses. Smadel, Rivers & Pickels (1939) estimated the dry weight of the vaccinia virus particle to be $5{\cdot}34 \times 10^{-15}$ g. The volume of a brick-shaped particle with dimensions $260 \times 220 \times 220$ mμ is $1{\cdot}25 \times 10^{-14}$ cm.^{3}, and the dry weight would be about $3{\cdot}6 \times 10^{-15}$ g., in fairly good agreement with the value of Smadel *et al.* The DNA content of purified vaccinia virus was given by Hoagland, Lavin, Smadel & Rivers (1940) as 5·6%, from which the amount of DNA/particle can be computed as $2{\cdot}0 \times 10^{-16}$ g. As far as we are aware, it has never been established previously whether pox viruses contain significant amounts of RNA. The analyses of Hoagland *et al.* (1940) were not decisive, since they were based on nucleic acid isolated with the use of hot aqueous sodium hydroxide, which would have eliminated by hydrolysis any RNA present. The problem is of interest since the presence of two different nucleic acids in an organism might be taken as a criterion for rejecting it as a true virus; the pox viruses are the largest of the true viruses on this definition, as our results show. We have concluded that pox virus particles have about $2{\cdot}6 \times 10^{-16}$ g. DNA and no RNA.

Adenovirus. There is general agreement that adenoviruses contain DNA. Morgan, Howe, Rose & Moore (1956), who examined adjacent thin and ultrathin sections of cells infected with adenovirus type 3 by histochemical techniques and electron microscopy, showed that parts of the nucleus packed with virus particles were Feulgen-positive. Armstrong & Hopper (1959) found that adenovirus-infected cells stained with acridine orange had accumulations of large amounts of DNA-containing material in nuclei; similar histochemical results were obtained with partially purified adenovirus suspensions by Epstein, Holt & Powell (1960). Valentine (1959) concluded from electron scattering of adenovirus type 5 particles stained with uranyl acetate that the nucleic acid content of the particles was high, perhaps as high as 50%. Our results confirm that adenovirus type 5 contains DNA and no RNA, the nucleic acid content of the particles being of the order of 30%.

Papilloma virus. Taylor, Beard, Sharp & Beard (1942) reported that the Shope papilloma virus had 9% DNA. Subsequent electron micrographs have shown that this virus is spherical, with a diameter of about 45 mμ (Williams, Kass & Knight, 1960). The calculated DNA molecular weight is thus about 7 millions. Recently Watson & Littlefield (1960) found the papilloma virus to have 8% DNA. Isolated DNA proved to be double-stranded and to have two components (21S and 28S) on ultracentrifugation (7 and 14 million). The larger component seems too large for a virus particle of molecular weight 45 million and 8% DNA. Hence it is either an artefact (perhaps a dimer) or most particles in the preparations examined were incomplete, with low DNA contents.

Tipula iridescent virus. This virus multiplies in the cytoplasm of susceptible insect cells. It is icosahedral, with a diameter of 130 mμ and contains DNA (Williams & Smith, 1957). Histochemical studies have shown considerable accumulations of DNA-containing material in the cytoplasm of cells infected with the virus (Armstrong & Niven, 1957). The amount of DNA recovered in our preparations corresponded to $2{\cdot}3 \times 10^{-16}$ g./particle, representing 16% of the dry weight; no RNA was present.

Nuclear polyhedral viruses of insects. The nuclear polyhedral viruses of insects so far analysed contain DNA and no RNA. From the sizes and percentages of DNA given in Table 4 it can be calculated that the absolute contents are of the order of 10^{-16} g./particle.

Comment on DNA viruses

Table 4 shows that in a large number of DNA viruses (10 bacteriophages, 3 nuclear and one cytoplasmic insect virus, and 3 mammalian viruses) the absolute amount of nucleic acid/particle does not have a wide distribution but falls within the relatively narrow range of 0·6 to $2{\cdot}6 \times 10^{-16}$ g. It would be remarkable if this agreement were fortuitous. It seems that the minimum amount of genetic information normally required for replication of a DNA virus is much greater than that required for a RNA virus. This would be expected because of the greater structural complexity of the larger DNA viruses. It is uncertain how much of the DNA in these viruses is transmitted to daughter virus particles in the form of a single molecule. The experiments of Levinthal (1956) and of Stent, Sato & Jerne (1959) suggest that about 40–60 % of the DNA of T-even phages is transferred to progeny virus particles in the form of molecules containing not less than 15 % of the amount of DNA/phage particle (i.e. having a molecular weight of 20 millions).

Cheng (1959) suggested that there is a widespread mechanism which limits chain lengths of DNA molecules to give basic molecular weights of about 4 millions. However, when the DNA of T-even phages is carefully prepared (Davison, 1959) it has a high sedimentation coefficient (S_{20w} = 60, corresponding to a molecular weight approaching 100 millions). High molecular-weight DNA is readily broken down to smaller units by turbulence and high shear gradients such as those produced by pipetting solutions. Hence reports of low molecular weights of extracted DNA preparations are open to doubt on technical grounds.

There are two known exceptions to the rule that DNA viruses have nucleic acid contents of molecular weight 60 to 260 millions. One is the small phage ϕX174, which has a single-stranded DNA and a nucleic acid content of the same order as that in the RNA viruses (about 2 millions). The other is the rabbit papilloma virus, which probably has a double-stranded DNA content of the order of 7 millions. The same is probably true of the structurally related, but not identical, polyoma virus which produces multiple tumours when inoculated into mice and hamsters. The successful initiation of infection with DNA extracts of all of these three viruses has been reported (Di Mayorca, Eddy, Stewart, Hunter, Friend & Bendich, 1959; Sekiguchi, Taketo & Takagi, 1960; Ito, 1960). The infectivity was abolished by deoxyribonuclease but not by ribonuclease; neither enzyme has any effect on intact virus. The preparation of infective DNA from these viruses is evidently not due to a single-stranded configuration, since papilloma virus DNA is double stranded. However, for technical reasons mentioned above, the preparation of intact DNA molecules of very high molecular weight such as occur in most viruses is likely to be much more difficult than the preparation of intact molecules of molecular weight 2–7 millions.

The origin of DNA viruses has been discussed elsewhere (Luria, 1953). There is enough evidence to consider seriously the view that they may be pieces of chromosomal DNA that have become differentiated so that cell-to-cell transfer occurs

efficiently. The location of prophage on the host chromosome, and phenomena such as transduction and lysogenic conversion, are powerful arguments in support of this view in the case of bacteriophages. No strictly analogous phenomena are known for the DNA viruses of insects and vertebrates, but other points of resemblance with phages suggest that they may have had similar origins. The fact that some DNA viruses, such as vaccinia and Tipula iridescent virus, multiply in the cytoplasm of infected cells is not a decisive argument against this view. Enzymes required for DNA synthesis are present in cytoplasm (Kornberg, 1960), and the site in the cell where DNA replication takes place may depend simply on the location of the template or 'primer'.

If it be accepted that members of the rickettsia and psittacosis-lymphogranuloma groups have evolved from bacterium-like precursors, the question arises whether the true viruses might have arisen by further modification of organisms of this type. The change would have involved the loss (simultaneously or successively) of either DNA or RNA, of mucopeptide cell walls and penicillin sensitivity, of a tetracycline-sensitive protein synthetic pathway and sulphonamide-sensitive metabolism. The mode of reproduction would have had to change from binary fission to one in which nucleic acid and virus protein are independently synthesized and then reassembled (Schäfer, 1959; Cohen, 1957). A radical series of metabolic changes of this type might conceivably have occurred, but it seems simpler to regard the DNA and RNA viruses as modified cell constituents.

Comment on RNA viruses

There has already been much discussion of the fact that all RNA viruses so far analysed have nucleic acid molecular weights of the order of 2 million. Infectious RNA extracted from tobacco mosaic and foot-and-mouth disease viruses have molecular weights of the same order (Gierer, 1960; Ströhmaier & Mussgay, 1959). In other words, all the RNA in these viruses appears to be present in a single polynucleotide chain containing some 6000 nucleotides. A single break in the polynucleotide chain produced by ribonuclease digestion, or modification of more than one base by nitrous acid treatment, is sufficient to abolish infectivity (Gierer, 1960). Hence the polynucleotide chain has to be transmitted intact to the recipient cell in order to reproduce itself and to initiate the synthesis of a virus-specific protein coat.

Microsomes from animal and plant tissues and ribosomes from bacteria also have RNA contents corresponding to about 2 millions (Palade & Siekevitz, 1956; Ts'o, Bonner & Vinograd, 1958; Gierer, 1958; Tissières & Watson, 1958). This coincidence has raised the question whether RNA viruses could have originated from cellular particles containing RNA. Available evidence seems rather against this view. The suggestion (Crick & Watson, 1956) that ribosomal particles might have the same structure as spherical viruses, with protein subunits regularly arranged around an RNA core, has not been borne out by recent observations on ribosomes. Furthermore, current work suggests that the structural RNA of ribosomes is not directly concerned with protein synthesis, but that a short-lived RNA intermediate serves as the 'messenger' carrying genetic information from nuclear DNA to protein (Brenner, Jacob & Meselson, 1961; Gros *et al.* 1961).

There is presumptive evidence that RNA can also carry information from one

cell to another. Extracts containing RNA added to bacteria have been reported to initiate the synthesis of enzymes not present in the recipient cells (Kramer & Straub, 1956). Preparations containing RNA added to embryonic cells are stated by Niu (1958) to bring about specific differentiation. These effects are lasting, which suggests that the RNA may be replicated in the cells rather than being a short-lived messenger. It is but a small further step to a virus RNA molecule that is self-perpetuating and capable of redirecting the protein synthetic mechanisms of recipient cells to form specific products. These, in turn, might aggregate to coat the RNA and protect it from ribonuclease and other degradative agents. Transfer of RNA from cell to cell, normally a chance event with a low probability of influencing the recipient cell, might have been 'streamlined' by natural selection so that in viruses it has become a devastatingly efficient process. The reason why a minimum RNA molecular weight of 1 to 2 millions is apparently required for this process is not clear. This is considerably more than the amount of RNA which would be required for specifying the amino-acid sequences of protein subunits on current coding hypotheses. Hence it would seem that virus RNA has to subserve other functions during the course of self-replication. Evidence is accumulating that some tobacco mosaic mutants have normal protein subunits (Wittmann, 1960), which would be in accordance with this interpretation.

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The Structure of Bacteriophage ϕR

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SUMMARY

Bacteriophage ϕR was examined in the electron microscope by the metal shadowing and negative-contrast techniques. The particle was a very small icosahedron and appeared to possess an extremely small tail. Some information was also obtained about the structure of the protein coat.

INTRODUCTION

The history of bacteriophage ϕR which attacks *Escherichia coli* and *Salmonella typhi* was described by Kay (1961). It resembles the minute bacteriophages S13 (Zahler, 1958) and ϕX-174 (Sinsheimer, 1959) which are themselves closely related and it possesses in common with them a single-stranded form of deoxyribonucleic acid in contrast to the double helical type found in the larger phages. Hall, Maclean & Tessman (1959) examined ϕX-174 in the electron microscope by shadowcasting and by the negative-contrast technique of Brenner *et al.* (1959). They found that virus particle was an extremely small polyhedron 248 ± 10 Å across with a pentagonal symmetry and no apparent tail. Little information was obtained about the three-dimensional structure of the particle. The present paper describes information on bacteriophage ϕR obtained by using a variety of different electron microscope specimen preparation techniques.

METHODS

Preparation of the phage suspension. The methods used for the growth and purification of the phage used in this work were described by Kay (1962). In the final stage of purification, the phage was eluted from a diethylaminoethyl cellulose column in 0·2 M-ammonium acetate at pH 7·4. The eluate contained $2{\cdot}9 \times 10^{12}$ plaque-forming particles/ml. This preparation had the advantage of being in a completely volatile buffer; as can be seen in Fig. 4 which is typical of many fields examined, it contained very little material other than phage particles.

Electron microscope preparation techniques

The phage particles were examined by the following methods: platinum metal shadowing, platinum + carbon shadowing (Bradley, 1959), a negative contrast method with lanthanum acetate; a negative contrast method with uranyl

acetate (Bradley & Kay, 1960). Details are as follows. Platinum shadowing was carried out by drying a suspension of phage in ammonium acetate on freshly cleaved mica (Hall & Litt, 1958) followed by shadowing with platinum. The shadowing layer was backed with a film of evaporated carbon and the composite film floated on a water surface from which it was picked up on support grids. Most of the phage particles remained attached to the mica so that a replica was in fact obtained.

Platinum + carbon shadowing was carried out according to the methods of Bradley (1959) using platinum + carbon rods of average composition 60 % carbon and 40 % platinum at a working distance of 5 cm. with a 2 mm. intermediate aperture and an evaporating angle of 2:1. The particles were first mounted on freshly cleaved mica as with the platinum shadowing. The platinum + carbon film was floated on water and then mounted on grids.

The preparation with lanthanum acetate was carried out by mixing an equal volume of phage suspension (in 0·2 M-ammonium acetate) with a 1 % (w/v) solution of lanthanum acetate (pH 4·5). The resulting suspension (pH 5·2) was dried on a carbon-coated grid.

In the case of uranyl acetate, a similar procedure was used, a 1 % (w/v) solution of uranyl acetate (pH 3·5) replacing the lanthanum acetate. The mixture was at about pH 4·2.

RESULTS

Platinum-shadowed particles

Micrographs similar to those of Hall *et al.* (1959) were obtained. Plate 1, fig. 1, shows that, in spite of a particularly granular shadowing layer, the six knobs observed by these authors were present in the ϕR specimen. Most of the particles were orientated so that one knob is surrounded by five others in regular pentagonal array. A number of particles exhibited a completely different orientation, however; one of these is illustrated in Pl. 2, fig. 2, where eight knobs can be seen. This was not observed by Hall *et al.* (1959) who found that all their particles were in the first orientation. The appearance of the particle is exactly consistent with an icosahedron having twelve subunits. (An icosahedron is a regular solid body which has twenty equiangular triangular faces, twelve apices and a 5:3:2 symmetry.) In the first case (Pl. 1, fig. 1) it is viewed down its five-fold symmetry axis, and in the second (Pl. 1, fig. 2) down its two-fold axis. This implies that the phages are standing either on an apex or an edge. It will also be noticed that there is an appreciable gap between subunits, particularly in Pl. 1, fig. 2, indicating that, in all probability, the knobs do not represent the single subunits of a protein coat.

Platinum + carbon shadowing

A heavily shadowed replica was prepared so that the combination of high contrast and sharp shadows obtained under such conditions would provide information about the geometrical form of the phage particle. Smith & Williams (1958) first used the analysis of shadow shape to determine the form of a virus particle. The same specimen was shadowed from two directions so that each particle cast two shadows. By comparing the shadow shapes with those obtained from models illuminated from different directions, it was possible to show that the virus in question, *Tipula* iridescent virus, was an icosahedron. It is not in fact necessary to shadow from two

directions, since a particle orientated suitably gave unmistakable characteristic shadow shapes. This fact was also recognized by Harrison & Nixon (1960) in a study of small plant viruses.

The heavily shadowed preparation of phage φR did not show up the knobs seen with the platinum-shadowed particles, because they were obscured by the thick shadowing film. A few particles did, however, exhibit the shadow shape shown in Pl. 1, fig. 3, the flat-topped one being typical of an icosahedron lying on a face, and the pointed one also lying on a face, but differing by 60° in orientation with respect to the shadowing direction. By far the greater majority of particles exhibited pointed shadows, since the preferred orientation was the same as with the platinum shadowed preparation, there being no difference in the method of deposition onto the mica substrate.

Particles shadowed more lightly with platinum + carbon showed up the knob structure, and also, in this particular replica, large areas of regularly packed phages were found (Pl. 2, fig. 4). It will be noticed that the packing is not truly hexagonal (the angle between the lines made by the particles is 108° instead of 120°) as would be expected with icosahedra. A likely explanation is that some distortion had taken place during drying; the significance of this will be discussed later.

Particles embedded in lanthanum acetate

Particles embedded in lanthanum acetate are illustrated in Pl. 2, fig. 5. There appears to be no preferential staining of the deoxyribonucleic acid or protein coat, in fact true 'negative staining' was achieved. The first most obvious point about this preparation is that there is no sign of the knobs seen in the shadowed specimens. The particles exhibit a well-defined hexagonal outline, their edges being straight and the angles sharp. The hexagonal outline is consistent with an icosahedron viewed down either its two- or three-fold symmetry axis. The rather coarse background of lanthanum acetate precludes the direct visualization of any small protein subunits.

Particles embedded in uranyl acetate

It can be seen from Pl. 3, fig. 6, that the hexagonal outline is apparent when uranyl acetate is used but that is not so obvious as in the lanthanum acetate preparation. It is clear that the staining mechanism with uranyl acetate is different and that some positive-staining has taken place in certain parts of the phage. The majority of the particles in Pl. 3, fig. 6, are outlined by a dark line; within this is a pale band, and the centre is noticeably darker than the general background. The large knobs found with the shadowed particles are absent, but a number of phages possess black specks arranged in the same way (Pl. 3, fig. 6, arrowed; Pl. 3, fig. 7).

None of the micrographs studied so far has shown any regularly arranged subunits as demonstrated by Horne, Brenner, Waterson & Wildy (1959) with adenovirus, though in a very few cases, as in Pl. 3, fig. 8, some indication of organization appeared.

It has been thought up to now that this type of phage possesses no tail. The appearance of a small protrusion on a number of particles was therefore unexpected. One is shown in Pl. 2, fig. 5, and a few are discernible in Pl. 3, fig. 6, but those in

Pl. 3, fig. 9, are very clear and a figure can be given for their length, namely about 50 Å. More evidence in favour of the phage possessing a tail, or at least a unique point capable of combining with the host bacterium, is given in the preceding paper (Kay, 1961).

Size measurements

The average diameter of the phage ϕR was measured from micrographs calibrated with monodisperse suspensions of Dow polystyrene latex. The size of these lattices is known to within 10 %, the diameters being 1880 Å and 880 Å. The size of the phage was measured in four different ways, first from shadowed specimens (with sharper shadows than those shown here) using shadow length and the width of the shadow, secondly from the lanthanum acetate preparation, and thirdly from the uranyl acetate preparation. The results are as follows:

(1) From shadow widths:	335Å
(2) From shadow lengths:	295Å
(3) From the lanthanum acetate preparation:	300Å
(4) From the uranyl acetate preparation:	275Å

It is considered that the error due to the shadowing layer in (1) and (2) is small, but in all cases it is not reasonable to expect to measure to an accuracy beyond the limitations of resolution imposed by both specimen and microscope. These factors, together with the limitation of the latex itself, mean that an accuracy of better than 10 % is not possible. Thus the final size indicated from the above figures is 300 ± 30 Å This is rather larger than the figure given by Hall *et al.* (1959) for phage ϕX-174.

DISCUSSION

There seems no doubt that phage ϕR has the form of an icosahedron, but the number of subunits which compose the protein coat is at present uncertain. The origin of the knobs found in the shadowed specimens is difficult to ascertain, though they do not represent single subunits. There are, in fact, 12 of these knobs, arranged in the 5:3:2 symmetry of an icosahedron and it is likely that they are in some way related to the packing either of the deoxyribonucleic acid core or of the protein coat. The fact that a monolayer of particles does not show the hexagonal packing expected of icosahedra strongly suggests that distortion has taken place during drying and this might well cause the partial collapse of the particle, thus producing the knobs at the apices. It is interesting to compare the shadowed particles with the plant viruses studied in the same way by Harrison & Nixon (1960). These viruses are the same size and shape as phage ϕR, but they do not show any apical knobs, confirming that it is not preferential granulation of the shadowing material which causes the knobs.

The interpretation of the micrographs of uranyl acetate-stained phage (Pl. 3) is made difficult because of the dual properties of the strain. There is evidence that true negative staining has taken place, because the particles are surrounded by a dark layer, but, since the centres of the particles are also darker than the background, it is believed that the deoxyribonucleic acid within the particles has been positively stained. The dark specks seen in some of the particles indicate the presence of uranium and cannot be confused with the background granularity because of their greater contrast. They could be due to the negative stain penetrating be-

tween the subunits, or positive staining of the subunits, or the stained nucleic acid showing between the subunits. More information on the behaviour of uranyl acetate is required to settle this matter.

There seems little doubt from the evidence of univalency that the tail shown in Pl. 2, fig. 5, and Pl. 3, figs. 6 and 9, is genuine. Though it is extremely small, there is no reason why this tail should not carry out the functions of absorption and injection of the phage deoxyribonucleic acid. There is adequate space in the tail for a hollow core down which the nucleic acid could pass, especially as it is a single strand and not a double one.

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Bacteriophage ϕR shadowed with platinum; ×170,000.

Fig. 2. Bacteriophage ϕR observed down its two-fold symmetry axis; platinum shadowed; ×330,000.

Fig. 3. Bacteriophage ϕR heavily shadowed with platinum + carbon showing the characteristic shadows of an icosahedron; ×330,000.

PLATE 2

Fig. 4. Particles of bacteriophage ϕR arranged in a nearly hexagonal array, platinum + carbon shadowed; ×157,000.

Fig. 5. Bacteriophage ϕR embedded in lanthanum acetate; ×330,000.

PLATE 3

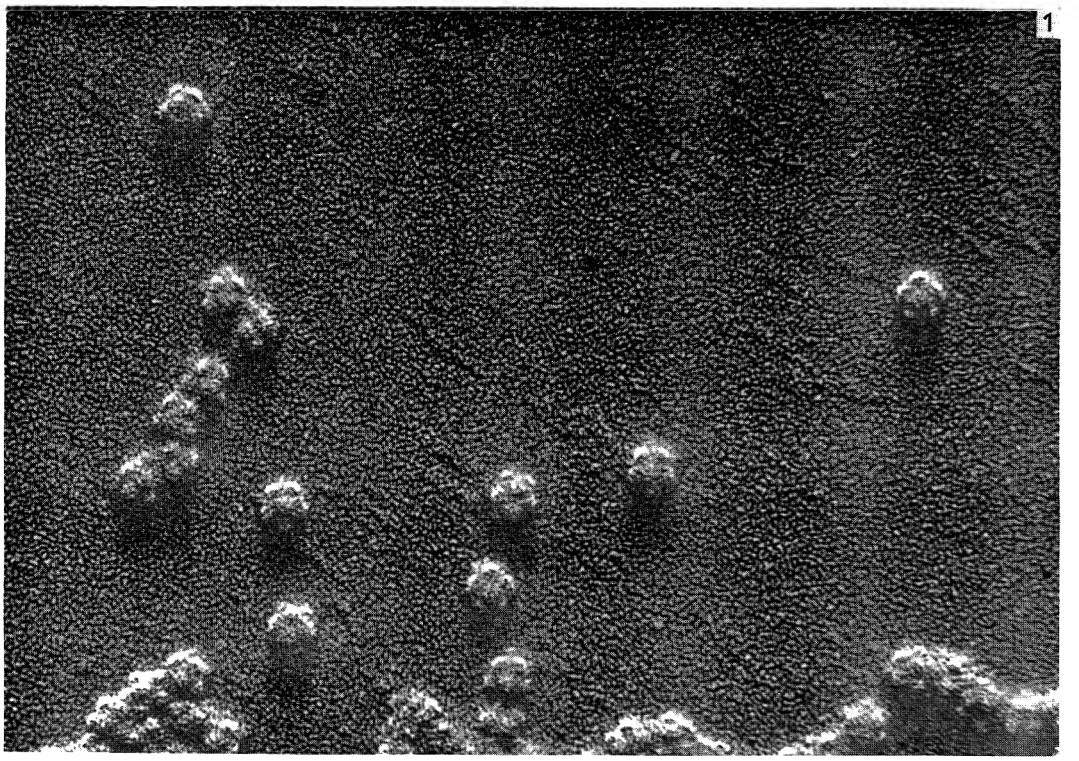
All the micrographs in this plate are of particles prepared in uranyl acetate.

Fig. 6. Particles clearly showing hexagonal outline; ×270,000.

Fig. 7. A single particle showing possible subunits; ×330,000.

Fig. 8. Some organization is visible on the arrowed particles; ×415,000.

Fig. 9. Three particles clearly showing the presence of a tail; ×330,000.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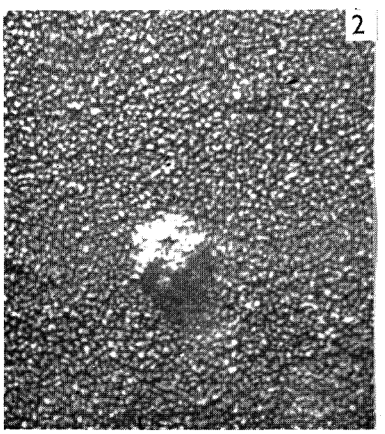
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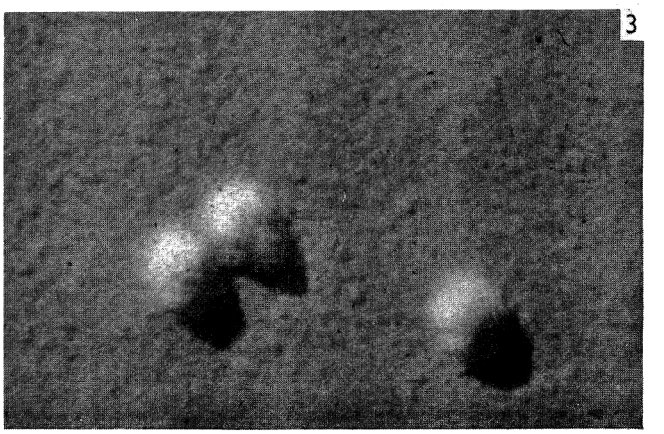
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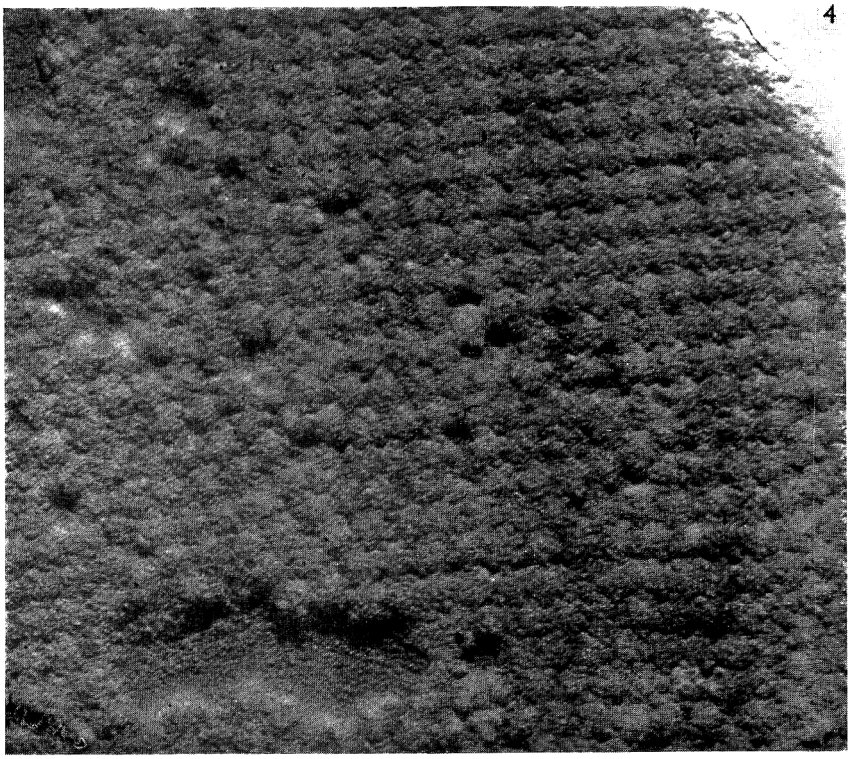

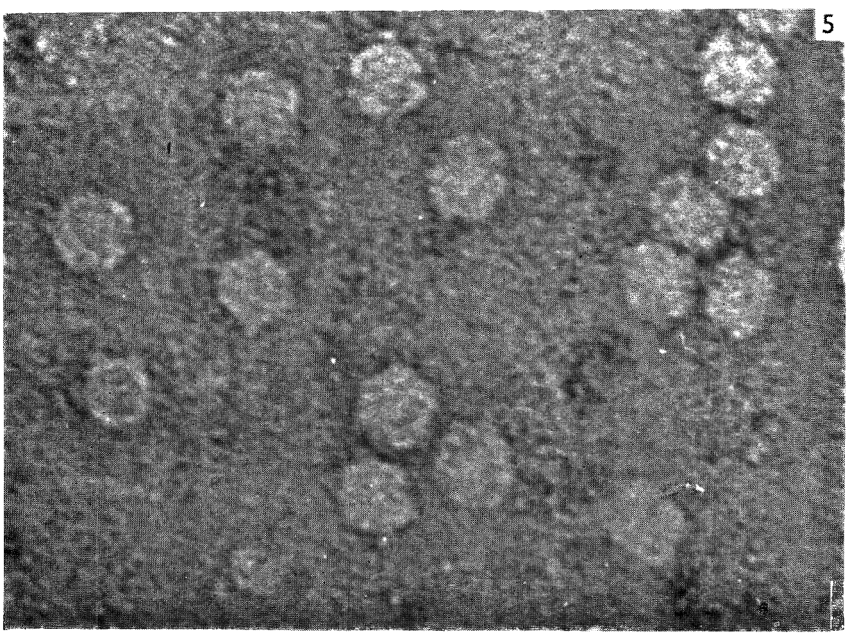

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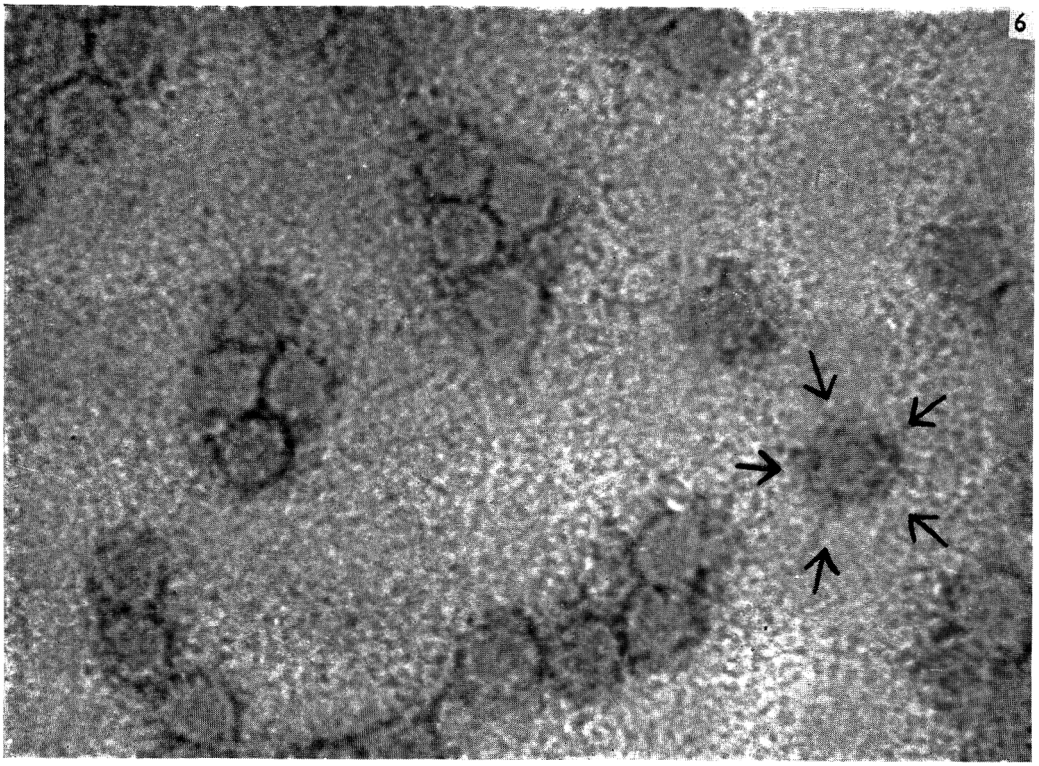
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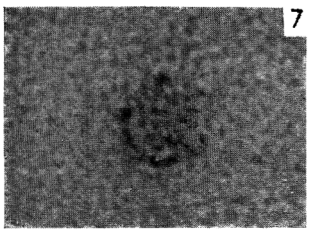
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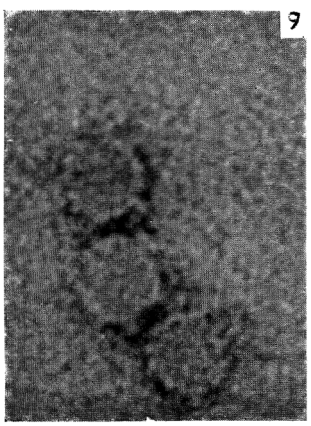
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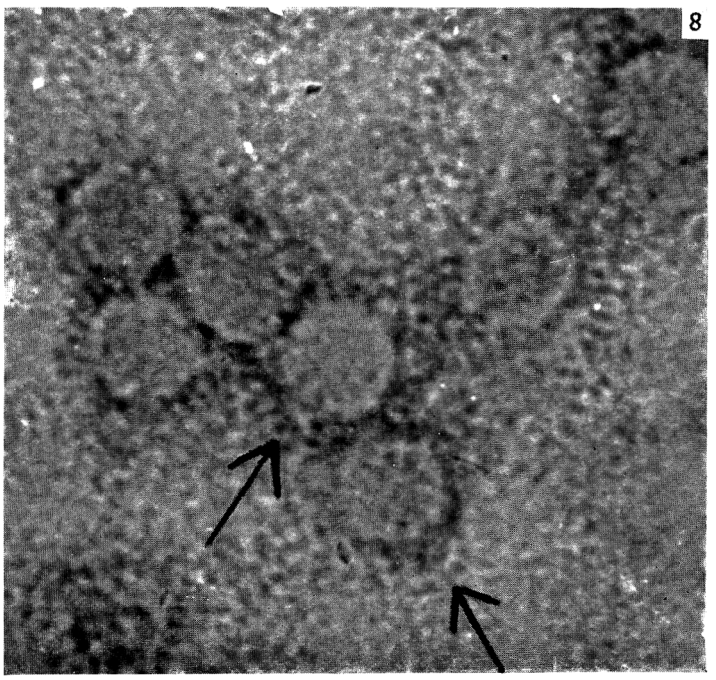
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The Nucleic Acid Composition of Bacteriophage ϕR

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SUMMARY

Bacteriophage ϕR was prepared and purified in sufficient quantity to determine the composition and base ratios of its nucleic acid. The ratios are such that the complementary double-stranded structure usually found in deoxyribonucleic acid (DNA) seems unlikely to be present. This observation and the accessibility of the DNA of phage ϕR to the action of formaldehyde support the view that phage ϕR, like phage ϕX-174 which it resembles, carries its nucleic acid in a single strand.

INTRODUCTION

Bacteriophage ϕR has been in our collection for some years. From electron micrographs taken in 1954 it was known to be very small in size and appeared to be tail-less. It was studied by Fildes (1954) and by Tucker (1961), when it was referred to as phage anti-R. Sinsheimer (1959), working with the very small bacteriophage ϕX-174, found that certain unusual properties of its nucleic acid could be accounted for by assuming a single-stranded instead of a double-stranded structure. This discovery prompted an investigation of the nucleic acid composition of phage ϕR to determine whether it, too, was of the single strand type.

Bacteriophage ϕX-174 resembles another coliphage, S13, in several ways; a comprehensive comparison between the two was made by Zahler (1958). A specimen of coliphage S13 was kindly made available by Dr K. Burton (Department of Biochemistry, University of Oxford) and this was compared with phage ϕR. Although the two phages have an entirely different history it has nevertheless proved difficult to differentiate them on grounds of host range alone. Bacterial strains showing well-marked differential sensitivity to these phages are rare and the results depend somewhat on the divalent metal ion content of the test media. Zahler (1958) found similar difficulties in distinguishing between ϕX-174 and S13. Phages ϕR and S13 can however be shown to differ immunologically even more markedly than do phages ϕX-174 and S13. It is therefore unlikely that ϕR is merely another isolate of ϕX-174 or S13 and it must be regarded as a different species of bacteriophage. The fine structure of phage ϕ R is of interest. Purified specimens of the phage made as described in this paper have been examined in the electron microscope. The results are described in a subsequent paper (Kay & Bradley, 1962).

METHODS

Bacteriophages and host bacteria. Bacteriophage ϕR was received from the late Dr A. Felix in 1953. He described it as an anti-rough phage, meaning that it lysed only the 'rough' stains of *Salmonella typhi* that he was studying. It was carried by a strain of *S. paratyphi* A isolated in Egypt in 1948 and had been maintained at first on *S. paratyphi* strain A59R and later on *S. paratyphi* strain 6SR. For the purpose of the present work it was found more convenient to grow the phage on *Escherichia coli* C (referred to as coli C) which was kindly supplied by Dr K. Burton (Department of Biochemistry, University of Oxford), who also supplied a specimen of phage S13.

Assay of phage ϕR. The phage was assayed by the two-layer method described by Adams (1950). The Petri dishes were kept at 37° for only 3 hr., by which time the plaques were countable. Further incubation allowed the plaques to grow to a large size, fuse and become uncountable.

Growth and purification of phage ϕR. Some difficulty was found in growing this phage to sufficiently high titre for purification. The rate of lysis of an infected culture depended on the divalent metal ion concentration, but unfortunately rapid lysis always resulted in low phage titres. A medium was devised containing peptone (Evans, 1 % (w/v)) and NaCl (0·5 % (w/v)). It was decalcified by boiling and filtering at pH 9·1 and was then readjusted to pH 7·6. Cultures of coli C were grown in 1 l. batches in 5 l. flasks rotated at 37° (Kay, 1959). At a population density of 4×10^8 bacteria/ml. phage was added to give a ratio of 1:1. Lysis took place slowly over 5 hr., giving phage titres of about 10^{11} plaque-forming units (pfu)/ml. When maximum clearing had occurred the phage was purified by a procedure modified from that of Sinsheimer (1959). The phage was precipitated with ammonium sulphate (400 g./l.) in the presence of ethylenediaminetetra-acetic acid (EDTA; 0·0025 M) at 4° overnight. The precipitate was collected by centrifugation at 15000 *g* for 1 hr. The sediment was extracted in borate + EDTA buffer (pH 9·7) for 3 hr. at 4°, centrifuged and re-extracted with the same buffer. The extracts were pooled, dialysed against borate + EDTA buffer (pH 9·1) at 4° and again precipitated with ammonium sulphate (280 g./l.) in the cold. The precipitate was extracted twice in borate + EDTA buffer (pH 9·1) and centrifuged for 2 hr. at 5000 *g* to remove inactive material. The phage was then sedimented at 140,000 *g* for 1 hr., the supernatant fluid discarded and the phage pellet resuspended in 0·033 M-phosphate buffer (pH 7·6) containing 0·01 % (w/v) human serum albumin. The suspension was then treated with deoxyribonuclease (1 μg./ml.) and $MgSO_4$ (0·004 M) for 1 hr. at room temperature and then at 4° overnight. The phage was then sedimented at 140,000 *g* for 1 hr. and resuspended in 0·033 M-phosphate buffer (pH 7·6).

Thirty litres of lysate containing about 2×10^{15} phage particles were treated in this way and yielded 2×10^{14} active phage particles.

Column chromatography of phage ϕR. The product from the large-scale phage preparation described above was not subjected to further purification but was used entirely for the nucleic acid composition studies described later. Other preparations of phage on a smaller scale were made for different purposes and were purified by a combination of the above procedure and chromatography on a column of DEAE (diethylaminoethyl)-cellulose.

The DEAE-cellulose was prepared by washing, successively, in N-HCl, water, N-NaOH and again water. The pH of the DEAE-cellulose was adjusted to 7·4 with HCl and washing with water continued. The column (2 cm. diameter) was packed with the DEAE-cellulose suspended in 0·2 M-ammonium acetate (pH 7·4) to a height of 7·5 cm. The column was washed with ammonium acetate until the effluent was at pH 7·4 and then washed with 200 ml. 0·01 M-ammonium acetate (pH 7·4).

Phage for chromatography was prepared as described above, up to the first centrifugation at 140,000 *g*. The pellet was resuspended in 0·01 M-ammonium acetate (pH 7·4), centrifuged at 4,000 *g* to remove insoluble matter and applied to the column. Stepwise elution was carried out with ammonium acetate buffer (pH 7·4) at 0·04 M, 0·1 M, 0·2 M, 0·5 M and M. Six fractions of 6 ml. were collected at each concentration and assayed for phage. The light absorption at 260 mμ was also measured. The phage was eluted as a single band in 0·2 M-ammonium acetate. It was spread over two 6 ml. fractions and also contained most of the 260 mμ absorbing material. Recoveries of phage were about 90 %. Eluates containing 5×10^{12} pfu/ml. possessed no visible turbidity and were used for the electron microscopy described in a subsequent paper (Kay & Bradley, 1961).

Antiphage sera. Antisera to phages φR and S13 were prepared by intravenous injection in rabbits of 0·5 ml. phage suspension at weekly intervals. The phage suspensions used were an ammonium acetate eluate containing 10^{11} pfu/ml. in the case of φR and a centrifuged lysate containing 2×10^{11} pfu/ml. in the case of S13. The animals were bled one week after the last injection.

RESULTS

Host range of phages φR and S13

The two phages were tested by the cross-streak method against several coli and typhoid bacteria from our collection. In no case was it possible to demonstrate a difference in the host range of these phages. The cross-streak test is not a quantitative method, so the plaque-forming ability of the two phages was determined by counting plaques on several strains of bacteria. The titres found, expressed as % of those given on coli C, were: coli strain 518, 85 and 35, typhoid strain O 901 R, 50 and < 1, typhoid strain R 4, 1 and < 1 for φR and S13, respectively. These figures show that the two phages are definitely different but that the differences are only quantitative since all the bacteria were sensitive to both the phages at some concentration. A more clear-cut distinction was given by the reaction of the phages to antiserum.

Serological difference between phages φR and S13

The antiphage activity of the sera prepared against the two phages was determined by mixing the phages, at 10^3 pfu/ml., with ten-fold serial dilutions of the homologous serum, incubating at 37° for 30 min. and comparing the number of plaque-forming units remaining with those in the controls without serum. All dilutions were made in a solution of 1 % (w/v) peptone + 0·5 % (w/v) NaCl. Each antiserum inactivated about 50 % of the homologous phage at a dilution of 1/50,000. Both phages were then tested against both sera at a dilution of 1/20,000. The phage

remaining active was assayed at intervals of 5 min. for a period of 30 min. (Fig. 1). The phages were inactivated by the homologous antisera only and were therefore clearly different from one another.

Univalency of phage ϕR

Electron micrographs of phage ϕR (Kay & Bradley, 1961) show that the particle is an icosahedron and that it probably carries a small tail. This protuberance is so small that it could be regarded as merely a specialized area of the phage protein

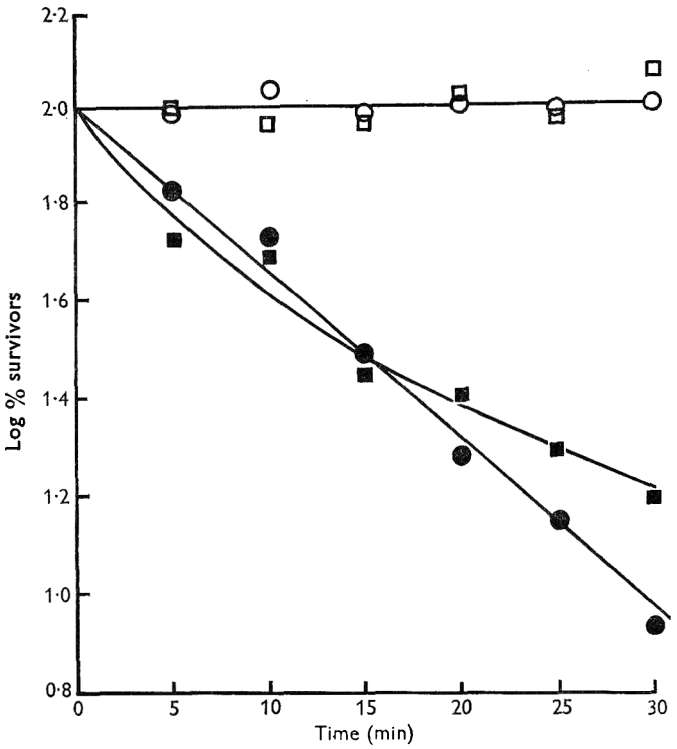

Fig. 1. Inactivation of phages ϕR and S13 by antiserum. Phage S13 and antiserum-S13, ●—●; phage S13 and antiserum-ϕR, ○—○; phage ϕR and antiserum-ϕR, ■—■; phage ϕR and antiserum-S13, □—□. Initial phage titre 1×10^3 pfu/ml. Antiserum dilution 1/20,000.

envelope. Nevertheless this phage must function in much the same way as other phages in that it must first absorb to its host cell and then transfer its DNA to the host through a perforation in the cell wall. These phage particles would therefore be expected to carry an organ concerned with absorption to the host and an enzyme which can digest the cell wall material at that site. If there were not a unique 'tail' on the phage in the accepted sense, and any part of the particle could act as an absorption site with an affinity for the bacterial receptor, then it might follow that a mixture of a great excess of phage particles with bacteria would lead to agglutination of the bacteria in the same way as certain animal viruses can give rise to haemagglutination. On the other hand, if under these circumstances no bac-

terial agglutination occurred, then it would appear that there was only one adsorption site on the particle and that the phage must have a single area capable of combining with the host cell. That is, it must have a single 'tail'.

In an attempt to resolve this matter a mixture of logarithmic-phase bacteria at 2×10^8/ml. and phage at 3×10^{11} pfu/ml. was made in a solution containing 1 % (w/v) peptone + 0·5 % (w/v) NaCl + 0·00025 M-$CaCl_2$. After 5 min. at 37° a portion of the mixture was concentrated and the free phage titre determined. It was found that 3 % of the phage was still free and that there must have been an average of

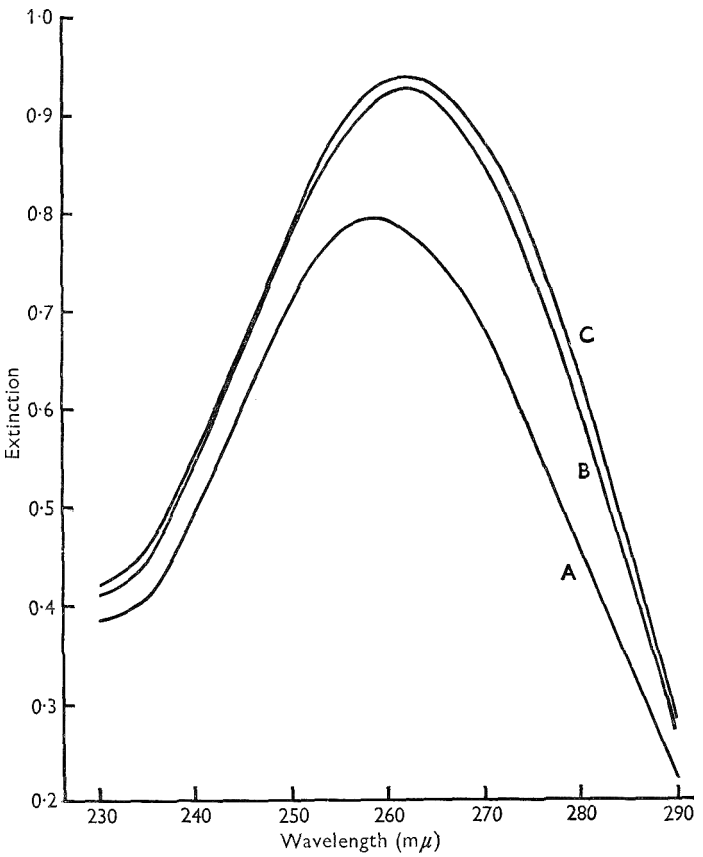

Fig. 2. Effect of formaldehyde (2 %, w/v) on the ultraviolet absorption of phage ϕR nucleic acid. A, immediately after mixing; B, after 4·5 hr.; C, after 24 hr.

1450 absorbed phage particles/bacterium. Part of the mixture was kept at 37° and part at room temperature. No gross agglutination was observed in either case, nor did the cells appear to be clumped together in a stained smear. Although this evidence is of a negative kind it can reasonably be taken to mean that phage ϕR possesses only one absorption site or 'tail'.

The purine and pyrimidine bases of phage ϕR nucleic acid

The nucleic acid was extracted by the procedure of Sinsheimer (1959) from a suspension of phage containing 2×10^{14} p.f.u/ml. prepared from 30 l. lysate as described in Methods. It was extracted into 2-methoxy-ethanol, precipitated with ethanol (60 %, v/v, in water) in the presence of a little sodium chloride, and washed successively in 60 %, 90 % (v/v) ethanol in water, absolute ethanol, then ether, and dried over phosphorus pentoxide. The bases were liberated from the nucleic acid by hydrolysis with 6 N-HCl at 100° for 3 hr. in a sealed Pyrex tube. The hydrolysate was evaporated to dryness *in vacuo* over KOH, taken up in water and chromatographed in propan-2-ol + HCl (Wyatt, 1951). Four spots containing the bases were located with ultraviolet irradiation and extracted into 0·1 N-HCl. The bases were identified as adenine, guanine, thymine and 'cytosine' from their ultra-violet absorption curves and their positions on the chromatogram. The 'cytosine' spot, which runs to the same place as hydroxymethylcytosine in propan-2-ol + HCl, was again chromatographed in propan-2-ol + ammonia (Hershey, Dixon & Chase, 1953) and found to run to the same place as authentic cytosine.

The molar ratios of the bases found were: adenine, 1·00; thymine, 1·41; guanine, 1·03; cytosine, 0·83. The comparable ratios for phage ϕX-174 nucleic acid (Sinsheimer, 1959) were 1·00; 1·31; 1·06; 0·82.

Effect of formaldehyde on phage ϕR nucleic acid

It was shown by Sinsheimer (1959) that the ultraviolet absorption of phage ϕX-174 nucleic acid was increased and the peak moved to a slightly longer wavelength by treatment with formaldehyde. This change, which does not take place with native deoxyribonucleic acid (DNA) from other sources, was explained by assuming a single-stranded structure for the phage ϕX-174 DNA, in which the amino groups of the bases would not be involved in hydrogen bonding to the bases in the complementary strand. The phage ϕR nucleic acid dissolved in 0·2 M-NaCl was treated with formaldehyde (2 %, w/v) at 37° in a glass-stoppered tube. The ultraviolet absorption curve was determined at the beginning, after 4·5 hr. and after 24 hr. (Fig. 2). The nucleic acid from phage ϕR showed the same hyperchromaticity as does that from phage ϕX-174. This observation and the lack of complementarity in the bases support the conclusion that the nucleic acid of phage ϕR is of the single-stranded variety.

DISCUSSION

Bacteriophage ϕR clearly belongs to the group of phages, now three in number, with phages ϕX-174 and S13. The distinguishing features of this group are the small size of the particle (245–300 Å diameter compared, for example, with 1000 × 700 Å diameter for coliphage T2) and the unusual single-stranded form of the DNA. In general, all the bacteriophages that have been examined, with the exception of the one containing ribonucleic acid (Loeb & Zinder, 1961) possess the 1:1 molar ratios of adenine: thymine and guanine: cytosine (or hydroxymethylcytosine) that are found in native DNA from all other sources (Evans, 1959). The new group of small bacteriophages does not show complementarity between its bases which, since they are not involved in hydrogen bonding, are accessible to formaldehyde.

In phage φR, as in phage φX-174, formaldehyde causes an increase in the ultraviolet absorption of the nucleic acid, an effect which is demonstrable with whole phage (Tucker, 1961), as well as with isolated nucleic acid, and which could form the basis of a screening test for bacteriophages possessing single-stranded nucleic acid.

Some of the electron micrographs of phage φR have shown what appears to be a very small 'tail' (Kay & Bradley, 1961) but the evidence is not conclusive. The behaviour of phage φR when absorbed in large numbers to bacteria supports the contention that there is only one absorptive site on the particle since bacterial agglutination does not take place, as it might be expected to do by analogy with haemagglutination by animal viruses. The evidence is of a negative kind and it would therefore be desirable to obtain further information about the absorption to and penetration of, the bacterial cell by phage φR in order to determine conclusively whether a 'tail' is present. Work is in progress with this end in view.

I wish to thank Miss Muriel Dangerfield for technical assistance.

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The Antibiotic Relationships of Some Free-living Bacteria

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SUMMARY

The sensitivity to antibiotics and the reactions to antibiotic-producing bacteria, streptomyces and fungi of a range of bacteria isolated from soil, leaves and forest litter showed broad differences between the Gram-positive and Gram-negative organisms, the fermentative and non-fermentative ones, and, to a lesser extent, between those of leaf origin and those of soil origin. These differences are not alone sufficient to explain the differences between the leaf flora and the soil flora. Other factors, including the capacity to produce antibiotics, are also important.

INTRODUCTION

The most distinct boundary separating natural populations of bacteria is that which divides the flora of the growing leaf (epiphytic flora) from the soil flora. Despite the very large populations which may obtain on leaves, such as those of pasture plants, in close proximity to the soil surface there is a rapid depletion of the epiphytic bacteria eluted into the soil so that few survive within the profile (Stout, 1960*b*). The biochemical and other physiological properties of the epiphytic bacteria do not appear to be very different from those of the common soil bacteria and it is difficult to attribute the failure of the epiphytic flora to colonize soil to their nutritional or other physiological shortcomings. One feature of the epiphytic flora, however, is the absence or virtual absence of streptomyces and the comparatively small numbers of fungi and spore-forming bacteria. These organisms are common in soil and are the most notable sources of antibiotic substances (Spector, 1957). It has therefore been thought possible that the extinction of the epiphytic flora in soil is due to microbial antagonisms and, in particular, to the effects of antibiotic substances. To test this hypothesis a range of bacterial isolates from various habitats (leaf, forest litter, soil) were tested against a selection of antibiotic substances produced by fungi, streptomyces and bacteria and against living fungi, yeasts, streptomyces and bacteria.

MATERIALS AND METHODS

The bacteria tested were isolated during recent years in the course of a survey of the grasslands, peats and forests of New Zealand. They have been classified as far as possible according to *Bergey's Manual* (1957). Further taxonomic and ecological details are given elsewhere (Stout, 1958, 1960*a*, *b*). Altogether 93 strains were used.

The antibiotic substances used are listed in Table 1 which also gives the source, the preparation, the diluent, and the concentrations tested. The bacterial and streptomycete antibiotics were generally readily soluble but some of the fungal antibiotics

Table 1. *List of antibiotics used, source, preparation, diluent, concentrations tested*

Antibiotic	Source	Preparation	Diluent	Concentrations tested
Polymyxin B sulphate	*Bacillus polymyxa*	Burroughs, Wellcome & Co. London, England	Water	100,000, 10,000, 1,000, 100 units/n
Tyrothricin (Gramicidin and Tyrocidine)	*Bacillus brevis*	Parke Davis & Co., Detroit, Michigan, U.S.A.	Ethanol	20,000, 10,000, 1,000, 100 μg./ml.
Bacitracin	*Bacillus subtilis* *Bacillus licheniformis*	Chas. Pfizer & Co. Inc., New York, U.S.A.	Water	500, 50, 5, 0·5 units/ml.
Streptomycin sulphate	*Streptomyces griseus* *S. bikiniensis* *S. mashuensis*	Glaxo Labs. (N.Z.) Ltd. Palmerston North, N.Z.	Water	10,000, 1,000, 100, 10 units/ml.
Neomycin sulphate	*Streptomyces fradiae* *Streptomyces* spp.	Glaxo Labs. (N.Z.) Ltd. Palmerston North, N.Z.	Water	1, 0·1, 0·01, 0·001 % (w/v)
Spiramycin	*Streptomyces ambofaciens*	May & Baker, Ltd., Dagenham, England	Methanol	10,000, 1,000, 100, 10 units/ml.
Oleandomycin phosphate	*Streptomyces antibioticus*	Chas. Pfizer & Co. Inc., New York, U.S.A.	Water	1,000, 100, 10, 1 units/ml.
Tetracycline hydrochloride	*Streptomyces* spp.	Chas. Pfizer & Co. Inc., New York, U.S.A.	Water	1,000, 100, 10, 1 units/ml.
Erythromycin lactobionate	*Streptomyces erythreus*	Abbott Labs., North Chicago, Illinois, U.S.A.	Water	5, 1, 0·1, 0·01 % (w/v)
Penicillin G (sodium salt)	*Penicillium notatum* *Penicillium* spp. *Aspergillus* spp.	Glaxo Labs. (N.Z.) Ltd., Palmerston North, N.Z.	Water	100,000, 10,000, 1,000, 100 units/n
Patulin	*Penicillium* spp. *Aspergillus* spp.	Wellcome Research Laboratories London, England	Water	2, 1, 0·1, 0·01 % (w/v)
Griseofulvin	*Penicillium griseofulvum* *Penicillium* spp.	Development Dept., Glaxo Labs. (N.Z.) Ltd., Palmerston North, N.Z.	Ethanol	2, 1, 0·1, 0·01 % (w/v)
Gliotoxin	*Aspergillus fumigatus*	Dr P. W. Brian, I.C.I. Ltd., Welwyn, Herts., England	Acetone and ethanol	2, 1, 0·1, 0·01 % (w/v)
Viridin	*Trichoderma viride*	Dr P. W. Brian, I.C.I. Ltd., Welwyn, Herts., England	Acetone and ethanol	2, 1, 0·1, 0·01 % (w/v)
Frequentin	*Penicillium frequentans*	Dr P. W. Brian, I.C.I. Ltd., Welwyn, Herts., England	Acetone	2, 1, 0·1, 0·01 % (w/v)
Mycophenolic acid	*Penicillium brevi-compactum* *Penicillium* spp.	Dr P. W. Brian, I.C.I. Ltd., Welwyn, Herts., England	Ethanol	2, 1, 0·1, 0·01 % (w/v)

were difficult to dissolve. Tests with acetone and ethanol showed that these solvents did not themselves inhibit growth in the concentrations used.

Broth cultures of the bacteria were prepared and used to inoculate agar plates which for most of the experiments consisted of tryptone (0·1 %), Difco yeast extract (0·1 %), agar (1·5 %), made up in distilled water. Potato carrot agar was used in experiments with the streptomycete plugs. This medium consisted of diced potatoes (20 g.) and carrots (20 g.), steamed in 1 l. distilled water for 20 min.; this was then filtered, made up to 1 l., agar added to 1·5 % (w/v) and autoclaved. Four filter paper disks (1 cm.) impregnated with the appropriate dilutions of the selected antibiotic were used for each plate. The total diameter of the zone of inhibition was measured after incubation for 24 hr. at 25° and summed for each plate.

The reproducibility of the technique was tested on several occasions with reasonably satisfactory results (Table 2). Consequently only a single plate was poured for each isolate tested. Strain variation (Table 3) did not appear to be important with

Table 2. *Results of duplicate plating*

Sum of the diameters of the four zones of inhibition for each plate in mm.

	Species and strain tested			
	P. fluorescens (C45)		*Aerobacter cloacae* (E6)	
	a	*b*	*a*	*b*
Antibiotic	Inhibition zone diam. (mm.)			
Polymyxin	49	47	49	58
Streptomycin	81	95	88	88
Tetracycline	31	40	21	33
Oleandomycin	0	0	15	23

Table 3. *Strain variation*

Sum of the diameters of the four zones of inhibition for each plate in mm. n.d. = not done.

	Species and strain tested				
	Chromobacterium lividum		*Bacillus cereus*		
Antibiotic	45/1/3	45/1/25	60/2/79	60/2/121	60/21/2
Polymyxin	35	29	61	n.d.	63
Tyrothricin	0	0	0	n.d.	30
Bacitracin	0	0	0	n.d.	20
Streptomycin	72	45	102	n.d.	102
Neomycin	81	25	47	45	52
Spiramycin	0	0	25	40	26
Oleandomycin	30	37	77	n.d.	65
Tetracycline	45	16	58	n.d.	51
Erythromycin	85	61	0	109	111
Penicillin	88	0	0	40	70
Patulin	65	50	10	58	70
Griseofulvin	0	0	0	0	10
Gliotoxin	0	0	87	142	111
Viridin	20	23	108	142	130
Frequentin	0	10	0	37	23
Mycophenolic acid	0	0	10	10	10
Mixed (Gliocladium)	25	25	46	53	30

the Gram-negative bacteria, e.g. Chromobacterium, but was more significant with the bacilli, particularly *Bacillus cereus*. The strains of *B. cereus* used were, however, as diverse as possible, one having been isolated as a smooth mucoid form, another as a pigmented form, and a third the more typical rough cream form.

For testing the inhibition of bacterial growth by other organisms two techniques were used, the plug method and the streak method. In the plug method the test organisms (fungi, yeasts, streptomyces) were grown on the soya bean agar plates (Smith, Gordon & Clark, 1952) for 14 days. Plugs of agar (0·6 mm. diameter) were then cut and placed on the bacterial plates (four to a plate) and the zone of inhibition measured in the same way as with the antibiotic disks. In the streak method soya bean agar plates were streaked with the test organisms (bacteria, yeasts) in four parallel lines and the plates incubated for 7 days. Loops of broth cultures of the different bacteria were then streaked between these initial inocula and the degree of growth arbitrarily assessed. Four degrees were recognized: no growth, slight growth, moderate growth, and good growth, which were scored, respectively, 0, 1, 2, 3. Neither of these techniques was wholly satisfactory, the plug method being superior to the streak method. The latter was used, however, principally for the bacteria for which the plug method was clearly unsuitable because of their rapid and spreading growth on the tryptone yeast-extract agar. In the event, both methods provided a better index of antibiotic-producing organisms rather than of antibiotic-sensitive organisms. This was particularly so with the streak method, for it was observed with the control plates that certain of the bacterial strains tested grew very poorly on the soya bean agar plates while others grew very well.

RESULTS

Inhibition of bacterial growth by antibiotic substances

Comparison of the efficacy of the different antibiotic substances is qualified by their differences in purity, solubility, and the concentrations tested. Of the seventeen antibiotics tested only griseofulvin proved virtually ineffective. The results are given in Table 4. These show:

(*a*) The Gram-positive bacteria (Micrococcus, Sarcina, Bacillus, Nocardia) were more sensitive than the Gram-negative bacteria (Pseudomonas, Xanthomonas, Aeromonas, Flavobacterium, Achromobacterium, Chromobacterium, Aerobacter, Escherichia and Serratia).

(*b*) The aerogenic fermenters (Aeromonas, *Flavobacterium* sp. Type IV, Serratia, Escherichia and *Bacillus polymyxa*) tended to be relatively insensitive, but *Escherichia coli* appeared to be more sensitive than the other Gram-negative aerogenic fermenters.

(*c*) The Gram-negative bacteria of leaf origin (Xanthomonas, Flavobacterium) were generally more sensitive than those commonly found in soil (Pseudomonas) but the dominant leaf taxon *Flavobacterium* Type I was no more sensitive than the pseudomonads or the aerogenic fermenters.

Inhibition of bacterial growth by streptomycete and fungal plugs

The pattern of bacterial sensitivity to these inhibitory moulds and streptomyces was similar to that of the antibiotics. Of the Gram-negative bacteria, only the flavobacteria showed inhibition, the pseudomonads and aerogenic fermenters were

Medium: peptone yeast extract agar. Figures given are the sum of the diameters of the four zones of inhibition for each plate in mm. Where more than one strain was used the average for the taxon is given.

Bacteria tested. Taxon and type	No. of strains	Ecological habitat	Bacterial antibiotics				Streptomycete antibiotics							Fungal antibiotics								T
			1	3	3	Av.	4	5	6	7	8	9	Av.	10	11	12	13	14	15	16	17	Av.	
Pseudomonadaceae																						
Pseudomonas aeruginosa	1	Parasite	41	0	0	14	30	60	40	10	37	21	33	20	30	10	10	10	10	10	10	14	
P. fluorescens I	1	Soil	48	0	0	16	83	64	0	0	29	32	35	17	30	10	59	25	10	0	21	22	
P. fluorescens II	2	Soil	46	0	0	15	90	67	10	0	52	33	42	15	25	0	0	10	0	0	10	8	
Pseudomonas sp. III	6	Soil, litter	38	0	0	13	67	65	10	0	68	40	42	46	40	0	38	40	5	0	26	24	
Pseudomonas sp. IV	3	Soil, litter	34	0	10	15	37	44	22	3	74	21	34	69	54	5	11	15	5	0	11	21	
P. chlororaphis V	1	Soil	58	10	10	26	62	45	0	0	12	45	21	0	25	10	0	10	0	0	0	6	
Pseudomonas sp. VI	1	Soil	39	0	10	16	40	60	40	40	57	22	43	10	23	10	0	10	0	0	0	7	
Xanthomonas sp.	2	Leaf	38	31	37	35	45	45	39	61	131	35	59	114	60	0	42	34	23	16	16	38	
Aeromonas sp.	1	Soil	38	0	0	13	58	60	0	10	25	23	20	10	26	0	38	24	10	0	0	14	
Rhizobiaceae																						
Chromobacterium lividum	2	Soil, leaf	42	0	0	14	59	53	0	38	73	30	42	88	58	0	0	22	5	0	25	25	2
Achromobacteriaceae																						
Achromobacter sp.	6	Soil	18	15	40	24	37	48	21	27	125	46	51	74	61	2	46	50	20	15	38	38	4
Flavobacterium sp. I	2	Leaf	10	10	0	7	34	42	13	0	64	14	28	47	49	0	10	10	27	0	30	22	2
Flavobacterium sp. II	4	Leaf	44	30	42	39	62	44	32	47	126	40	59	142	35	0	38	35	20	0	13	35	4
Flavobacterium sp. III	5	Leaf	22	68	0	30	105	49	21	10	106	59	58	107	55	0	48	38	10	25	26	38	4
Flavobacterium sp. IV	1	Leaf	63	0	0	21	73	58	0	17	90	56	49	45	39	0	47	30	10	0	10	23	3
Enterobacteriaceae																						
Escherichia coli	1	Parasite	61	0	0	20	85	57	0	10	64	46	44	43	102	0	37	34	10	0	30	32	3
Aerobacter cloacae	5	Soil, leaf, litter	43	0	0	14	66	46	13	20	63	22	38	45	42	0	34	27	3	0	10	20	2
Serratia sp.	2	Soil	54	0	0	20	85	57	0	5	58	21	34	20	52	0	28	20	0	0	10	16	2
Micrococcaceae																						
Micrococcus spp.	8	Leaf	51	17	38	35	86	52	29	34	130	42	62	107	38	3	94	97	21	3	34	50	5
Sarcina sp.	1	Litter	13	0	89	34	103	40	61	112	145	57	86	142	61	0	94	102	11	0	25	54	6
Bacillaceae																						
Bacillus megaterium	3	Soil	29	30	33	31	60	70	65	77	134	64	78	109	54	0	116	85	22	0	42	54	5
B. cereus	5	Soil, leaf, litter	49	20	17	29	65	30	30	71	110	56	60	55	46	3	113	127	20	10	43	52	5
B. mycoides	2	Soil, leaf, litter	75	30	25	43	82	40	32	117	150	109	88	81	103	0	96	162	41	10	53	68	7
B. polymyxa	3	Soil	38	13	0	17	50	49	25	55	115	58	59	118	55	0	66	47	22	0	42	44	4
B. circulans	2	Soil	27	30	120	59	75	51	10	52	142	65	66	145	131	0	180	168	43	90	99	107	8
B. pantothenticus	1	Soil	27	30	20	26	60	46	37	58	112	41	59	40	65	0	138	118	42	10	32	56	5
B. sphaericus	3	Soil	15	30	24	23	0	50	64	43	115	31	51	114	52	0	81	88	35	0	44	52	4
Actinomycetaceae																						
Nocardia sp. I	3	Soil, litter	22	30	41	31	45	68	40	34	127	44	60	128	63	0	62	20	37	29	26	46	4
Nocardia sp. II	3	Soil	25	30	52	32	39	41	27	22	135	37	50	132	51	0	95	58	22	24	33	52	4
Myxococcaceae																						
Myxococcus sp.	1	Soil, litter	10	30	98	46	30	120	95	50	139	70	84	120	17	0	56	60	20	10	69	44	5

insensitive. Inhibition occurred most commonly with the Gram-positive bacteria although one strain of Micrococcus proved insensitive to the streptomycete plugs, and a strain of Sarcina and the majority of the Bacillus species were unaffected by the moulds. The results are given in Tables 5 and 6. Most of the streptomyces tested showed inhibitory activity against some bacterial strains although the two strains of *Streptomyces hygroscopicus* (Table 5, nos. 4 and 5) were appreciably more effective than the others. The pattern of bacteria sensitivity resembled that shown by the streptomycete antibiotics.

Table 5. *Inhibition of bacterial growth in potato carrot agar by 8 streptomycete plugs grown on soya bean agar*

All the Pseudomonodaceae (nine strains), Rhizobiaceae (one strain) and Enterobacteriaceae (four strains) tested showed no inhibition. Figures given are the sum of the diameters of the four zones of inhibition for each plate in mm. Where more than one strain was used the average for the taxon is given.

Bacteria tested. Taxa	No. of strains	Streptomycete plugs used								Total inhibition
		1	2	3	4	5	6	7	8	
Achromobacteriaceae										
Flavobacterium sp. I	3	0	0	0	0	0	0	0	0	0
Flavobacterium sp. II	1	9	9	8	18	13	8	9	0	74
Flavobacterium sp. III	1	8	9	8	20	26	0	8	0	79
Flavobacterium sp. IV	1	0	0	0	0	0	0	0	0	0
Flavobacterium sp.	3	7	3	2	15	15	0	2	0	44
Flavobacterium sp.	1	0	0	0	0	0	0	0	0	0
Flavobacterium sp.	2	0	0	0	0	0	6	0	0	6
Flavobacterium sp.	1	10	12	0	0	26	8	10	6	71
Micrococcaceae										
Micrococcus sp.	1	17	8	9	22	19	0	6	6	87
Micrococcus sp.	1	0	0	0	0	0	0	0	0	0
Bacillaceae										
Bacillus cereus	2	0	3	0	14	13	0	0	3	33
B. mycoides	1	0	0	0	18	18	0	0	0	36
Actinomycetaceae										
Nocardia sp.	1	12	0	0	18	22	0	0	0	52
Nocardia sp.	1	0	0	0	26	16	0	8	0	50
Total inhibition		63	43	27	151	168	22	43	15	532
No. taxa inhibited		6	3	4	8	9	3	6	3	10

The few fungi which showed inhibitory activity included the most commonly recorded antibiotic-producing genera, namely *Aspergillus*, *Penicillium*, *Gliocladium*, *Fusarium* and particularly *Trichoderma* (see Spector, 1957). *Trichoderma viride* was easily the most effective. *Aspergillus fumigatus*, *Gliocladium roseum*, *Penicillium frequentans*, *Fusarium culmorum* and *Cylindrocarpon radicicola* were effective against one or two bacterial strains. The dominant soil fungi, such as *Rhizoctonia* (Thornton, 1958), and the fungi found on leaves (*Pithomyces chartarum* syn. *Sporidesmium bakeri* and *Stemphylium*) were ineffective.

Inhibition by *Trichoderma viride* generally resembled the pattern of sensitivity to viridin but was not absolutely identical. Thus *Bacillus mycoides* and *B. circulans*, the two organisms most sensitive to viridin, were also sensitive to *T. viride*, but other viridin-sensitive bacilli, such as *B. cereus* and *B. megaterium*, were not.

Table 6. *Inhibition of bacterial growth in tryptone yeast extract agar by plugs of fungi grown on soya bean agar*

All the Pseudomonodaceae (5 strains), Rhizobiaceae (1 strain) and Enterobacteriaceae (3 strains) tested showed no inhibition. Figures given are the sum of the diameters of the four zones of inhibition for each plate in mm.

Bacteria tested. Taxa	No. of strains	Fungal plugs used 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	Total inhibition
Achromobacteriaceae																		
Flavobacterium sp. I	1	0	0	0	0	0	14	0	0	0	0	0	0	0	0	0	0	14
Flavobacterium sp. II	1	0	0	0	0	0	14	0	0	0	0	0	0	0	0	0	0	14
Achromobacter sp.	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Micrococcaceae																		
Micrococcus sp.	1	0	0	0	20	0	45	0	0	0	0	0	0	0	0	0	0	65
Micrococcus sp.	1	0	0	0	0	0	20	0	0	0	0	0	0	0	0	0	0	20
Sarcina sp.	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bacillaceae																		
Bacillus megaterium	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B. cereus	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B. mycoides	1	0	0	0	0	0	18	0	0	0	0	0	0	0	0	0	0	18
B. polymyxa	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B. circulans	1	0	0	0	14	0	45	0	0	0	0	0	20	24	0	0	0	103
B. sphaericus	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Actinomycetaceae																		
Nocardia sp. I	1	0	0	0	0	0	12	0	0	0	0	0	0	0	0	0	0	12
Nocardia sp. III	1	0	0	0	0	0	26	0	0	0	0	0	0	0	0	0	0	26
Myxobacteriaceae																		
Myxococcus sp.	1	20	0	0	0	22	0	0	0	0	0	0	22	0	0	0	0	64
Total inhibition		20	0	0	34	22	194	0	0	0	0	0	42	24	0	0	0	336
No. taxa inhibited		1	0	0	2	1	8	0	0	0	0	0	2	1	0	0	0	9

Fungi tested: *Aspergillus fumigatus* (1), *Penicillium nigricans* (2), *Penicillium janthinellum* (3), *Penicillium frequentans* (4), *Gliocladium roseum* (5), *Trichoderma viride* (6), *Absidia glauca* (7), *Mucor hiemalis* (8), *Zygorhynchus moelleri* (9), *Mortierella elongata* (10), *Rhizoctonia* sp. (11), *Cylindrocarpon radicicola* (12), *Fusarium culmorum* (13), *Fusarium avenaceum* (14), *Pithomyceschartarum* (15), *Stemphylium* sp. (16).

The first is associated with decaying herbage and the last three with growing herbage. The remainder are soil fungi.

Medium: soya bean agar. Measure of growth: 0, no growth; 1, poor growth; 2, moderate growth; 3, good growth.

Bacteria tested. Taxa	No. of strains	Degree of growth by inocula																Control
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	
Pseudomonadaceae																		
Pseudomonas aeruginosa	1	1	2	1	3	1	2	1	2	2	2	2	2	2	2	0	2	2
P. fluorescens I	1	0	0	0	0	0	0	1	0	1	0	0	0	0	1	1	0	1
Pseudomonas sp. III	1	0	0	0	2	0	1	0	0	1	0	0	0	2	0	2	0	1
Xanthomonas sp.	1	2	3	0	0	1	1	2	2	2	1	2	2	0	2	0	2	2
Aeromonas sp.	1	3	3	3	3	1	2	3	3	3	3	3	2	3	3	2	3	3
Achromobacteriaceae																		
Flavobacterium sp. I	1	2	2	0	2	0	2	2	0	2	2	2	1	1	2	2	2	2
Flavobacterium sp. II	1	2	3	0	3	1	0	2	3	1	2	3	2	0	2	0	3	2
Achromobacter sp.	1	0	1	0	0	0	0	1	1	0	0	0	1	0	0	0	2	1
Rhizobiaceae																		
Chromobacterium lividum	1	2	3	1	3	1	1	1	2	2	1	1	1	2	3	1	2	2
Enterobacteriaceae																		
Escherichia coli	1	3	3	3	3	1	3	3	3	3	3	2	2	3	3	0	3	3
Aerobacter cloacae	1	3	3	3	3	1	3	3	3	2	2	2	2	3	3	1	3	3
Serratia sp.	1	3	3	3	3	1	2	2	3	3	2	2	1	3	3	3	3	3
Micrococcaceae																		
Micrococcus sp.	1	0	3	1	0	0	0	1	1	1	0	0	1	0	1	0	2	2
Micrococcus sp.	1	1	2	1	3	1	1	2	2	2	2	2	1	2	2	0	2	2
Sarcina sp.	1	0	2	0	0	0	0	0	0	1	0	0	1	0	0	0	1	2
Bacilllaceae																		
Bacillus megaterium	1	1	2	0	0	0	2	2	2	2	1	1	1	0	2	0	2	2
Bacillus cereus	1	0	2	0	0	0	1	2	0	2	0	1	1	0	2	0	2	2
Bacillus mycoides	1	1	2	0	0	1	2	2	2	1	0	1	1	1	1	0	2	2
Bacillus polymyxa	1	2	2	2	2	1	3	3	2	2	1	1	1	2	3	1	2	3
Bacillus circulans	1	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	1
Bacillus sphaericus	1	0	2	1	0	0	1	1	1	1	0	2	1	0	1	0	1	2
Actinomycetaceae																		
Nocardia sp. I	1	1	2	1	1	2	2	2	2	2	2	2	2	1	2	0	2	2
Nocardia sp. III	1	1	2	1	0	1	2	2	2	2	2	2	1	2	3	0	2	2
Myxobacteriaceae																		
Myxococcus sp.	1	0	2	0	0	0	0	1	1	0	0	0	2	0	0	0	2	2
Total	24	28	49	21	31	14	31	39	37	38	27	31	30	27	41	13	45	49

Similarly, Micrococcus but not Sarcina was inhibited by the fungus though both were sensitive to viridin; and the flavobacteria and nocardias were inhibited by the fungus though they were not particularly sensitive to viridin.

The same bacterial strains used in the fungi tests were also tested against eight yeasts (*Cryptococcus laurentii*, *C. albidus*, *Rhodotorula marina*, *Candida curvata*, *C. humicola*, *Hansenula saturnis*, *Trichosporon cutaneum*, *Sporobolomyces roseus*). The results were inconclusive. There was no evidence of inhibition on the streak plates but on the plug plates some bacteria (Xanthomonas, Aeromonas, Flavobacterium) showed a very slight zone of inhibition round the plug. Since the yeasts grew on the sides of the plug and on to the surface of the plate after inoculation the significance of this very limited inhibition is not clear. It may have been due to competition for nutrients, but since Aeromonas, a facultative anaerobe, was affected it could not have been due in this case at least to induced anaerobiosis under the plug.

Inhibitory effect of bacterial streak plates

The results are shown in Table 7. Some bacteria (e.g. the aerogenic fermenters) grew well while others (e.g. some pseudomonads and bacilli) grew very poorly on the soya bean agar pre-streaked with bacteria. This was due partly to the limited nutrient status of the agar and partly to its desiccation during the period of incubation when the bacterial streaks were growing. Consequently, these plates did not give a clear picture of the relative sensitivity of the different taxa tested. However, the results did indicate that certain of the streaked bacteria, namely *Bacillus polymyxa*, *Aeromonas*, and *Pseudomonas chlororaphis*, did possess appreciably greater inhibitory powers than the other strains. *Aeromonas* and *P. chlororaphis* also proved to be two of the most effective bacteria in inhibiting the growth of yeasts (di Menna, 1961).

DISCUSSION

The present evidence does not support the thesis that sensitivity to antibiotics is a major factor in the discontinuous distribution of free-living bacteria. The principal taxon found on pasture leaves, often in enormous numbers and commonly absent from soil, namely *Flavobacterium* Type I (see Stout, 1960*b*), is no more sensitive to these antibiotics than the common soil pseudomonads. Other minor leaf taxa, however, are more sensitive and consequently may be excluded from the soil by this factor. The most sensitive group are the aerobic spore-forming bacilli which are amongst the most widespread and numerous of soil organisms. Clearly other attributes are more important in determining the ecology of these bacteria. One such attribute may be the capacity to produce antibiotics (see Brian, 1957). In this respect the bacilli and nocardias, typical soil bacteria, are the most commonly recorded bacterial sources of antibiotics (Spector, 1957). In the present experiments both *Bacillus polymyxa* and *Pseudomonas chlororaphis* appeared to have some inhibitory effect on bacterial growth. This conforms to their known production of polymyxin and chlororaphin, respectively (Florey *et al.* 1949). It is interesting to note that polymyxin also inhibited the growth of *B. polymyxa*. A similar situation obtains with megacin and *B. megaterium* (Ivánovics & Nagy, 1958). A possible explanation of this curious anomaly, at variance with the fungal evidence (Brian,

1957), is that the antibiotic is produced during spore formation (Bernlohr & Novelli, 1959) and the spores, as distinct from the vegetative cells, may be unaffected.

Although *Pseudomonas fluorescens* is often dominant in the indigenous grassland soils of New Zealand (Stout, 1958) and in the exotic pastures developed from them (Stout, 1960*a*), *P. aeruginosa* has not been isolated from these soils. There does not appear to be any distinction in their sensitivity to antibiotics to account for this difference in distribution between the saprophyte and the parasite. Similarly, although *Escherichia coli* may be recovered from herbage and from soil in a grazed pasture it does not appear to persist in either habitat, despite its relative tolerance of antibiotics.

The suggestion that sensitivity to antibiotics may afford a useful taxonomic criterion (Shewan, Hodgkiss & Linton, 1954) receives some support from this study, though a wider range of strains of the individual taxa would need to be tested before it could be applied with confidence. Amongst suggestive results the complete insensitivity of both strains of *Bacillus sphaericus* tested to streptomycin at the concentrations used, was noteworthy, while some of the sharpest contrasts between different taxa were obtained with bacitracin and tyrothricin.

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Biochemical Genetical Studies on the Pathway of Sulphate Assimilation in *Aspergillus nidulans*

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SUMMARY

Mutant strains of *Aspergillus nidulans* requiring methionine for growth were isolated and their growth responses to inorganic and organic sulphur sources studied. It is suggested that methionine is synthesized in this mould from inorganic sulphate through sulphite, thiosulphate and cysteine. The mutant strains genetically blocked in the reaction from thiosulphate to cysteine could be divided into two genetically different groups by means of the heterokaryosis test; heterokaryons formed between these two groups were able to grow on sulphate as sole sulphur source because of the syntrophic action of two different types of nuclei. It was further shown that cysteine-S-sulphonate supported good growth of one of the two groups, whereas the other group showed no growth on this compound. It was concluded that the metabolic conversion of thiosulphate to cysteine involves cysteine-S-sulphonate as an intermediate. Mutant strains responded to sulphide in the same way as to thiosulphate. From this and other evidence, it is suggested that sulphide is utilized by way of thiosulphate.

INTRODUCTION

Despite numerous studies so far reported, no conclusive scheme has yet been presented for the metabolic pathway by which inorganic sulphate is converted to sulphur-containing amino acids in micro-organisms. Recent investigations have established that the first step of this assimilation involves the activation of sulphate by means of adenosine triphosphate and subsequent reduction of the active sulphate to sulphite (Wilson & Bandurski, 1958; Hilz & Kittler, 1958; Hilz, Kittler & Knape, 1959; Ragland, 1959). Three different pathways have, however, been suggested for the further fate of the sulphite sulphur.

In one of the three suggested possibilities it is postulated that sulphite directly reacts with pyruvate to form β-sulphinyl-pyruvate which is then converted to cysteine via cysteine sulphinate. This pathway has been suggested to be operative in *Aspergillus nidulans* (Shepherd, 1956). The formation in rabbit kidney of radioactive cysteine sulphinate from ^{35}S-labelled sulphite has also been reported (Chapeville & Fromageot, 1954).

In the second pathway, first suggested on the basis of evidence obtained with *Escherichia coli*, it is assumed that sulphite sulphur is reduced to the sulphide state before its introduction into the carbon chain of cysteine (Lampen, Roepke & Jones, 1947). Evidence for this pathway seems to be given by the finding that a pyridoxal phosphate-dependent enzyme occurs in yeast and catalyses the condensation of

hydrogen sulphide with serine (Schlossmann & Lynen, 1957). Little is, however, known of the mechanism by which sulphite is reduced to sulphide, except for the report that this reduction can be catalysed by a very unstable enzyme system in yeast which uses reduced triphosphopyridine nucleotide as hydrogen donor (Lezius, 1959). Working with enzyme preparations from yeast, Hilz *et al.* (1959) excluded thiosulphate as an intermediate in this reduction process.

In contrast to the above two pathways, it is suggested as a third possibility that sulphite is converted to thiosulphate and that the sulphur-carbon linkage is formed at this stage of sulphate reduction. This pathway was proposed by Horowitz (1950), based on observations with nutritional mutants of *Neurospora crassa*. It has further been suggested from studies with mutant strains of *Aspergillus nidulans* that thiosulphate reacts with serine to give cysteine-S-sulphonate as an intermediate in the biosynthesis of cysteine (Hockenhull, 1949). Shepherd (1956) also considered this possibility, but denied the involvement of thiosulphate in the main route of sulphate assimilation.

The present work was undertaken with the hope of throwing light on the problem of sulphate assimilation by using a biochemical genetical approach. Mutant strains of *Aspergillus nidulans* which grew with methionine as a nutrient component were used for this purpose. The results obtained convincingly indicate that cysteine-S-sulphonate is an obligatory intermediate in sulphate assimilation in *A. nidulans* and that thiosulphate is the form in which sulphate-sulphur combines with the carbon skeleton of cysteine in the assimilatory process. A preliminary report of this work was published elsewhere (Nakamura & Sato, 1960).

METHODS

Organism. A strain of *Aspergillus nidulans* IFO 5713 was supplied from the Institute for Fermentation, Osaka.

Media. The basal sulphur-deficient medium contained per l. deionized water: 30 g. glucose; 3 g. $NaNO_3$; 1 g. KH_2PO_4; 0·5 g. K_2HPO_4; 0·2 g. $MgCl_2$; 0·1 g. $CaCl_2.2H_2O$; 1 ml. trace element solution. This medium was at pH 6·6–6·8 before autoclaving. The trace element solution contained (mg./l.): $FeCl_3.6H_2O$, 1000; $CuCl_2.2H_2O$, 300; $MnCl_2.4H_2O$, 72; $(NH_4)_6Mo_7O_{24}.4H_2O$, 40; $ZnCl_2$, 4200. The basal medium was supplemented with various sulphur compounds (100 mg./l.) for use in nutritional tests. When thiosulphate, sulphide or cysteine-S-sulphonate was added as sulphur source, KH_2PO_4 was omitted and the amount of K_2HPO_4 was increased to 1·2 g./l. This modification resulted in an increase in pH of the medium to 7·4–7·6. Sulphite, thiosulphate, sulphide, cysteine-S-sulphonate and cysteine were separately sterilized by Seitz filtration and aseptically added to the steam-sterilized basal medium. The basal medium supplemented with sulphate, etc., will be designated simply as 'sulphate medium', etc. When solid media were needed, 17 g. purified agar/l. were added. Autoclaving was at 121° for 15 min.

The germination medium used for isolating mutants contained, per 800 ml.: 30 g. glucose, 3 g. $NaNO_3$, 0·2 g. $MgCl_2$, 0·76 g. KCl, 0·01 g. $FeCl_3.6H_2O$, 0·3 mg. $CuCl_2.2H_2O$, 1·0 g. Na_2SO_4. In germination experiments, 8 volumes of this medium were mixed with 2 volumes of conidiospore suspension in sterilized 0·1 M-phosphate buffer (pH 7·0; containing 0·29 g. KH_2PO_4, 0·77 g. K_2HPO_4/200 ml.); this mixture was at pH 6·8–7·0.

Compounds. Sodium L-cysteine-S-sulphonate was prepared by a modification (Nakamura, unpublished) of the method of Kolthoff & Stricks (1951) and purified by chromatography on a Dowex-1 column. Choline sulphate was synthesized according to Schmidt & Wagner (1904). Sulphate, sulphite, thiosulphate and sulphide were all used as sodium salts. The cysteine, cystine and methionine used were all L-isomers.

Isolation of mutants. Conidiospores of *Aspergillus nidulans* were collected from freshly grown slope cultures on the sulphate medium and suspended in 0·1 M-potassium phosphate buffer (pH 7·0). The suspension was shaken for 2 hr. to break the spore chains and filtered through gauze into Petri dishes. The filtered spore suspension (5 mm. deep) was then exposed to ultraviolet radiation (10 W. Toshiba UV lamp, 100 V.) from a distance of 40 cm. for 8 min. The survival rate under these conditions was 6·7 %. Twenty ml. of irradiated suspension was mixed with 80 ml. germination medium (containing Na_2SO_4 as sole sulphur source) and the mixture shaken at 32°. After shaking for 16, 20, 24 or 28 hr., cultures were filtered through 3 to 4 sheets of gauze to remove the mycelia of wild-type strains which had grown in this medium (Fries, 1947). The filtrate from the final filtration in which spores of mutant strains had been enriched was variously diluted and plated on the methionine agar medium. The colonies thus developed were picked on to methionine agar slopes to confirm their ability to grow on this medium. They were then transferred to the sulphate agar medium and those strains which failed to grow on the latter medium were selected as auxotrophic mutants. The stability of the mutants thus isolated was confirmed by monospore isolation and serial transfer to appropriate media. In all, 694 strains which had grown on the methionine medium were subjected to the screening as described and 14 stable methionine-requiring mutants were isolated (yield, 2·02 %).

Nutritional tests. Utilization for growth of various sulphur sources by the wild-type and mutant strains was studied as follows. Spores of each strain were separately inoculated into each of two sterile plugged tubes (15 × 120 mm.) each containing 15 ml. of basal medium supplemented with the sulphur compound to be tested. The tubes were incubated at 32° for 8 days and the growth examined. At least three independent sets of experiments were carried out to judge the growth response. Quantitative experiments were also made with cysteine-S-sulphonate as the sulphur source. The mycelia grown after desired periods of incubation were collected on pre-weighed sheets of filter paper, washed with water, and dried at 90° for about 4 hr. The dried mycelia, together with the filter papers, were then kept overnight over P_2O_5 *in vacuo* and the dry weight measured, the weight of filter paper being subtracted.

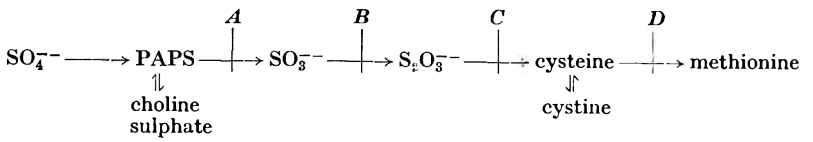
Production of heterokaryons. Heterokaryosis due to the vegetative hyphal fusion between two genetically distinguished mutants of a fungus (Pontecorvo, 1947, 1953) was extensively used in the present study to investigate metabolic pathways. The method used was based on the so-called limiting nutrition technique (Pontecorvo & Sermonti, 1954; Pontecorvo, Gloor & Forbes, 1954). Thick conidial suspensions (0·5 to 5×10^7 conidia/ml.) from the two mutants to be tested were well mixed and the mixture plated on the basal agar medium supplemented with 5 mg. (suboptimal) methionine + 100 mg. (sufficient) Na_2SO_4/l. In certain instances, mixed point inoculation was also made with the conidial suspensions at fixed

points on the agar medium. On incubating the mixed culture at 32°, the methionine suboptimally added to the medium permitted growth of both mutants and thus permitted the fusion between the homokaryotic hyphae of the component strains. When the genetic blocks in the two mutants used are located at different steps of the sole pathway from inorganic sulphate to methionine, the heterokaryon formed by the fusion must now be able to grow upon sulphate alone because of the syntrophic action of the two different types of nuclei in the heterokaryotic mycelium. When, however, the two component strains are genetically identical, such utilization of sulphate cannot be expected. The formation of heterokaryons in the mixed culture could thus be easily detected by observing the development of distinct large colonies on this medium. After incubation for 4–5 days, small mycelial tips of the heterokaryotic colonies were transferred to the sulphate medium and those which again showed good growth were selected as 'balanced' heterokaryons. In order further to confirm that the growth on sulphate alone was not due to contamination or to reversed mutation, these heterokaryons were examined by single conidium isolation. Since the conidium of *Aspergillus nidulans* is haploid and uninucleate, a single spore from a heterokaryon should bear the character of one or other of the component strains, except in the case of mitotic recombination. The rate of such recombination was, however, reported to be $1/10^6$ to 10^7 (Pontecorvo, 1953) and thus could be neglected. Conidiospores from the heterokaryon to be tested were therefore sown (about 500 conidia per Petri dish) on both the methionine and sulphate media and incubated at 32° for several days. When the sown conidia were taken from a true heterokaryon, active growth was observed only on the methionine medium and no colonies were formed on the sulphate medium. Sometimes one or two colonies were observed on the sulphate medium, but this might have been caused either by contamination of mycelial tips or by mitotic recombination. The formation of heterokaryons between two mutant strains was accepted only after these confirmations.

RESULTS

Response of mutants to various sulphur sources

In Table 1 are summarized the growth responses of the isolated 14 auxotrophic mutants (together with that of the wild-type strain) to various inorganic and organic sulphur sources. The wild-type strain utilized for growth all the sulphur compounds tested. It is clear from these results that, according to their nutritional requirements, the mutants can be classified into 4 groups which will be called groups *A*, *B*, *C* and *D* as indicated in Table 1. It appears that the simplest explanation for these findings as well as those reported earlier (Lederberg & Tatum, 1946; Hockenhull, 1949) may be provided by the following scheme of sulphate assimilation:



Here, the letters *A*, *B*, etc., indicate the sites of block in respective groups of mutants. Adenosine 3′-phosphate 5′-sulphato-phosphate (PAPS) is inserted in the

scheme to show the position of choline sulphate as elucidated by recent studies (Kaji & Gregory, 1959; Spencer & Harada, 1960). The role of sulphide in assimilation will be discussed later.

Table 1. *Growth responses of wild-type and mutant strains of* Aspergillus nidulans *to various sulphur sources*

		Sulphur sources							
Group	Strain	Na_2SO_4	Choline sulphate	Na_2SO_3	Na_2S	$Na_2S_2O_3$	Cysteine	Cystine	Methionine
		Growth response							
	Wild-type	+	+	+	+	+	+	+	+
A	954	−	−	+	+	+	+	+	+
B	574	−	−	−	+	+	+	+	+
C	562	−	−	−	−	−	+	+	+
C	611	−	−	−	−	−	+	+	+
C	649	−	−	−	−	−	+	+	+
C	721	−	−	−	−	−	+	+	+
C	793	−	−	−	−	−	+	+	+
C	831	−	−	−	−	−	+	+	+
D	519	−	−	−	−	−	−	−	+
D	523	−	−	−	−	−	−	−	+
D	795	−	−	−	−	−	−	−	+
D	845	−	−	−	−	−	−	−	+
D	950	−	−	−	−	−	−	−	+
D	984	−	−	−	−	−	−	−	+

+, Growth; −, no growth. For details see text.

Thiosulphate as intermediate

As already mentioned, it has been established that the reduction of sulphate to sulphite is an obligatory step in the microbial synthesis of sulphur-containing amino acids from sulphate. It has also been suggested that in *Neurospora crassa* methionine is formed from cysteine through the cystathionine pathway (Teas, Horowitz & Fling, 1948; Fling & Horowitz, 1951). The scheme proposed above for sulphate assimilation would, therefore, be acceptable if it were possible to show that cysteine is synthesized from sulphite via thiosulphate. A support for this may be found in Table 1 in which it is shown that all the 12 mutants which were unable to utilize thiosulphate (groups *C* and *D*) also did not grow on sulphite, and hence on sulphate, as sulphur sources. Furthermore, other evidence in favour of this possibility was obtained in heterokaryosis tests between thiosulphateless (group *B*) and cysteineless (group *C*) mutants. When strain 574 belonging to group *B* was subjected to hyphal anastomosis with 3 strains of group *C*, the formation of heterokaryons was detected in each pair; and the heterokaryons thus formed were capable of growing on sulphate as the sole source of sulphur.

These two pieces of evidence strongly suggest that thiosulphate is in fact a normal intermediate in the synthesis of cysteine from sulphate and sulphite by *Aspergillus nidulans*. But these results can also be equally well accounted for by assuming that the actual intermediate is not thiosulphate itself, but is a compound which can be readily derived from it. Further studies are, therefore, necessary to

decide which is really the case; but it seems fairly certain that either thiosulphate itself or a compound in equilibrium with it (e.g. enzyme-bound thiosulphate) lies on the normal pathway of cysteine biosynthesis in *Aspergillus nidulans*.

Incorporation of thiosulphate into cysteine

If thiosulphate can be admitted as a normal intermediate in sulphate assimilation, it is most probable that the sulphur-carbon bond in cysteine is formed at this stage of sulphate reduction. Hockenhull (1949) suggested that in *Aspergillus nidulans* thiosulphate reacts with serine to form cysteine-S-sulphonate as a preparatory step in the synthesis of cysteine. The validity of this hypothesis was studied by using the 6 strains of group *C* in which the conversion of thiosulphate to cysteine

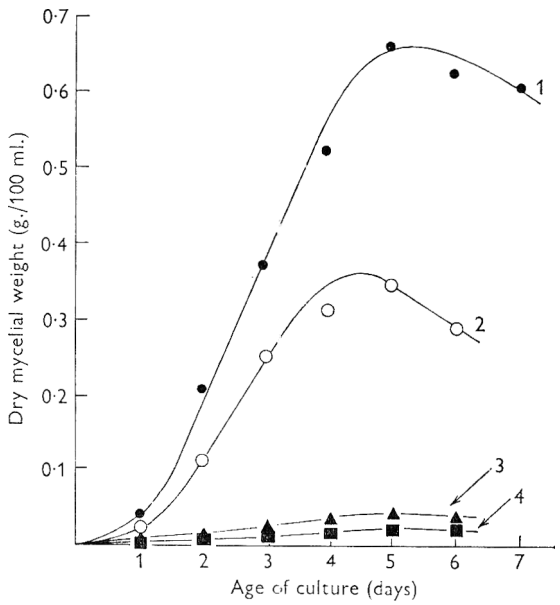


Fig. 1. Utilization of cysteine-S-sulphonate as sulphur source by several strains of *Aspergillus nidulans*. Experimental conditions as described in text. 1 = wild-type strain; 2 = strain 721 (*C*, cysteine-S-sulphonateless); 3 = strain 950 (*D*, methionineless); 4 = strain 793 (*C*, cysteineless).

Table 2. *Growth of wild-type strain and group C mutants of* Aspergillus nidulans *on cysteine-S-sulphonate as sulphur source*

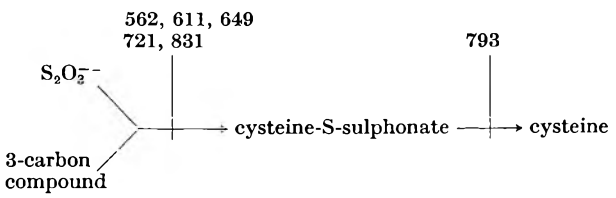
Average of four independent experiments. Yield of growth is expressed in terms of dry mycelial weight after 10 days cultivation in 15 ml. medium containing 100 mg. cysteine-S-sulphonate/l.

Strains						
Wild-type	793	562	611	649	721	831
Yield of mycelium (mg. dry weight)						
57·8	3·0	49·5	37·3	19·0*	39·3	40·8

* This strain showed rather poor growth even in a methionine medium.

is the site of genetic blocking. If this conversion actually involves cysteine-S-sulphonate as an intermediate, it might be expected that this step would be controlled by at least two genes. To examine this possibility, the 6 strains of group *C* were subjected to an allelism test by observing the production of heterokaryons on a medium containing sufficient sulphate and suboptimal amounts of methionine. It was thus found repeatedly that one strain (strain 793) of the 6 formed heterokaryons with any of the other 5 strains (strains 562, 611, 649, 721, 831). No signs of heterokaryosis were, however, detected with any pair of the latter 5 strains, indicating their genetical identity. Having thus confirmed that the block locus in strain 793 is different from that in the other 5 strains of group *C*, the ability of these mutants to utilize cysteine-S-sulphonate as sole sulphur source was then examined. As can be seen from Table 2 and Fig. 1, strain 793 was quite unable to utilize cysteine-S-sulphonate as sulphur source, whereas the other 5 strains as well as the wild-type strain grew well on this compound. Fig. 1 also shows that a strain of group *D* (strain 950) in which the reaction from cysteine to methionine is blocked did not utilize the sulphonate.

These results, together with those of Table 1, strongly suggest that strain 793 has a genetical block between cysteine-S-sulphonate and cysteine and that the other 5 strains of group *C* are deficient in the enzyme responsible for the condensation of thiosulphate with a 3-carbon compound to form the sulphonate. This situation is illustrated in the following scheme:



The nature of the 3-carbon compound is not yet clear, but it seems most likely that serine is the compound involved, in view of its structural relationship to cysteine-S-sulphonate. The observation reported by Hockenhull (1949) that the growth of thiosulphate-requiring mutants of *Aspergillus nidulans* on thiosulphate was enhanced by the addition of serine lends further support to this possibility.

Assimilation of sulphide

The results recorded in Table 1 indicate that the medium containing sodium sulphide as the sole sulphur source supported the growth of only those strains of *Aspergillus nidulans* which are capable of utilizing thiosulphate as sulphur source, i.e. the wild-type strain, strain 954 (group *A*) and strain 574 (group *B*). All the strains belonging to groups *C* and *D*, on the other hand, assimilated neither sulphide nor thiosulphate. These facts appear to exclude the possibility that sulphide and thiosulphate are used by mutually independent pathways. Instead, it seems likely that these two sulphur compounds are assimilated through a common metabolic pathway.

To elucidate further the nature of sulphide utilization, experiments were carried out to examine the behaviour of heterokaryons produced between different types

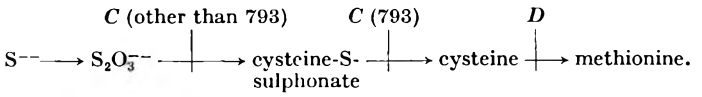
of mutants which by themselves are incapable of assimilating sulphide. The following three pairs of strains were arbitrarily chosen for this purpose:

562 (*C*, cysteine-S-sulphonateless)/950 (*D*, methionineless);

793 (*C*, cysteineless)/519 (*D*, methionineless);

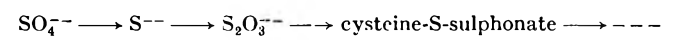
793 (*C*, cysteineless)/721 (*C*, cysteine-S-sulphonateless).

The results of the experiments clearly showed that all the pairs can form heterokaryons which can grow well, utilizing not only sulphate but also sulphide as sole source of sulphur. This indicates that these mutant strains acquire the ability to assimilate sulphide when their individually blocked reactions are complemented by heterokaryon formation. It is strongly suggested from these findings that sulphide, like thiosulphate, is utilized through the pathway involving cysteine-S-sulphonate, cysteine and methionine. The following scheme may therefore be presented for the pathway of sulphide utilization:

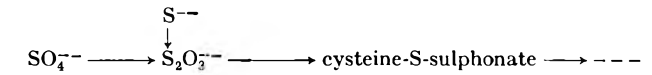


In this scheme the sequence sulphide → thiosulphate is adopted instead of thiosulphate → sulphide, since it is much more probable that thiosulphate, but not sulphide, is the immediate precursor of cysteine-S-sulphonate. It should be pointed out that the oxidative conversion of sulphide to thiosulphate has been reported to occur in mammalian tissues (Baxter, van Reen, Pearson & Rosenberg, 1958).

If the scheme just proposed be accepted, a further problem remains to be solved: is sulphide a normal intermediate in the assimilation of sulphate? If this be so, sulphate utilization may be represented as follows:



If, on the other hand, sulphide is not on the route from sulphate to thiosulphate, the following scheme may be more suitable to show the position of sulphide in the assimilation:



It is not yet possible from the evidence obtained in the present work to decide which is really the case. Further studies are needed to settle this point.

DISCUSSION

The results described in this paper provide evidence which indicates that in the assimilatory metabolism of *Aspergillus nidulans* inorganic sulphate is first reduced to sulphite and then converted to thiosulphate before its sulphur atom enters into direct combination with the carbon skeletons of sulphur-containing amino acids. It seems also fairly well established that thiosulphate reacts with a 3-carbon compound, probably serine, to form cysteine-S-sulphonate, which in turn is converted to cysteine. It may be expected that cysteine, once formed, can be either oxidized to cystine or further metabolized to methionine via the cystathionine pathway.

The mechanism by which sulphide is assimilated by *Aspergillus nidulans* is of interest in view of the studies reported by Schlossmann & Lynen (1957) who showed

that cysteine is synthesized in yeast by the direct condensation of sulphide with serine. Furthermore, Hilz *et al.* (1959) provided evidence that the reduction of sulphate to sulphide by yeast extracts proceeds through a pathway not involving thiosulphate as an intermediate. The data obtained in the present work suggest that sulphide is utilized by *A. nidulans* by way of thiosulphate and cysteine-S-sulphonate, although it is not certain that sulphide is an intermediate in the conversion of sulphate into thiosulphate. In any case, it seems unlikely that sulphide is the immediate precursor of cysteine in *A. nidulans*. These conflicting data concerning the role of sulphide appear to be explicable by assuming that different pathways in the process of sulphate assimilation are operative in *A. nidulans* as compared with yeast. The pathway functioning in *Escherichia coli* seems to involve sulphide as the immediate precursor of cysteine (Lampen *et al.* 1947) and thus resembles the pathway in yeast. The observations made by Horowitz (1950) and by Lederberg & Tatum (1946), on the other hand, suggest that *Neurospora crassa* assimilates sulphate via thiosulphate and hence cysteine-S-sulphonate, as does *A. nidulans*. It may be noted that in all the previous work with *A. nidulans* (Hockenhull, 1949; Shepherd, 1956) it has been assumed that cysteine is formed from thiosulphate by way of cysteine-S-sulphonate. Although Shepherd (1956) claimed that sulphate sulphur combines with the carbon chain at the sulphite stage in the normal assimilatory metabolism, it does not seem possible to explain the results described either in the present paper or in that of Hockenhull (1949) by assuming such a pathway.

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Isodensity Ultracentrifugation of Foot-and-Mouth Disease Virus in Caesium Chloride*

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SUMMARY

A rapid centrifugation method for purifying foot-and-mouth disease virus (FMDV) combined an isodensity separation below a moving zone separation in the same tube. Two ml. of crude infectious fluid were introduced over a 3 ml. nonlinear CsCl gradient in a swinging bucket rotor. This preformed gradient provided a gradual increase from density 1·0 to 1·3 g./ml. and a step from 1·3 to 1·6 g./ml. After centrifugation for 4 hr. at 37,000 rev./min. (120,000 *g*) and 4°, a 1 mm. wide light scattering zone was observed with type A virus near the bottom, clearly separated below debris extending from density 1·3 to the meniscus. The narrow light scattering zone contained (40 ± 12 %) of the FMDV infectivity and its CsCl isodensity was $1{\cdot}43 \pm 0{\cdot}01$ g./ml. Southern bean mosaic virus and bacteriophage ϕX174 behaved similarly and were useful as density markers. Virus suspensions concentrated an average of 8 fold retained (47 ± 16) % of their infectious units and were studied in the analytical ultracentrifuge directly. Dialysed concentrated virus revealed characteristic particles in an electron microscope. Exposure to concentrated impure CsCl decreases the stability of the infectivity to such an extent that in 40% CsCl its half-life is about 4 days.

INTRODUCTION

A rapid method was sought for concentrating and purifying the virus of foot-and-mouth disease (FMDV). Since this small virus (about 23 mμ diameter) is harvested at low concentration in the presence of large amounts of tissue debris, purification by differential centrifugation alone is very difficult. In addition to the time required, the repeated formation and resuspension of pellets leads to prohibitive losses of particles and infectivity. Meselson, Stahl & Vinograd (1957) demonstrated the efficacy of isodensity centrifugation in concentrated CsCl solutions for dense biological macromolecules, emphasizing that the field could be used to make the gradient. Previously, 'isopycnic' (Anderson, 1955) centrifugation had been used primarily for lipoproteins (reviewed by Lindgren & Nichols, 1960) and low density cellular components (reviewed by deDuve, Berthet & Beaufay, 1959). The use of a pre-

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formed gradient takes advantage of the fact that very large particles form bands before the CsCl or sucrose used for the density gradient reach equilibrium. Applications of isodensity methods using either 'field formed' or preformed gradients have been extensively reported for nucleic acids and viruses ('Spinco' Technical Reviews, 1960; Matthews, 1959; Brakke, 1961).

Recently, Matthews (1960) used a preformed linear gradient from 1·26 to 1·46 g./ml. with a small volume of turnip yellow mosaic virus layered on top. Centrifugation was only for 4 hr. at 32,000 rev./min. at low temperature. Light scattering fractions were dialysed to remove CsCl for further studies. He used both bucket and angle rotors. The non-linear preformed gradient method described here is a modification which uses a very low density near the top of the tube to permit a velocity separation followed by the formation of a band in the steep isodensity region below.

The use of moving zone techniques in a slight density gradient of sucrose or D_2O have been previously reported for FMDV (Strohmaier & Mussgay, 1959; Trautman, Savan & Breese, 1959; Breese, Trautman & Bachrach, 1960). Fortunately, the infectivity of the FMDV is not lost so quickly in the high CsCl concentration as to preclude measurement of its isodensity value. Once this was determined, various ways of utilizing isodensity methods in combination with other techniques became possible.

METHODS

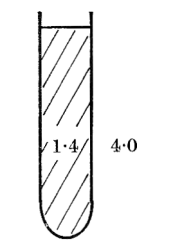
Virus source. FMDV, type A, strain 119 suspensions were used as collected from infected cultures of bovine kidney epithelial cells or after partial purification by methanol precipitation (Bachrach & Breese, 1958). They were titrated in 4 oz. prescription bottles by the plaque assay method (Bachrach, Callis, Hess & Patty, 1957). Vesicular fluid removed from the hind pads of guinea-pigs 24 hr. after inoculation with FMDV type A, strain GB was another source of virus. This virus had been highly adapted to guinea-pigs before receipt by this laboratory. It was titrated in suckling mice (Skinner, Henderson & Brooksby, 1952; Graves & Poppensiek, 1960) at decimal dilutions, with 10 mice per dilution. End-points were computed by the moving average method (Thompson, 1947) with a span of three. The assay error was in the order of 100 % for one standard deviation, which was somewhat greater than that of the plaque assay. In some experiments, starting materials were clarified by shaking with an equal volume of chloroform in the cold, centrifuging at low speed, and recovering the aqueous phase (Pyl, 1953; Bachrach & Breese, 1958). The southern bean mosaic virus (SBMV) was kindly supplied by Dr T. E. Cartwright, University of Pittsburgh, Pennsylvania, and the bacteriophage ϕX174 by Dr R. L. Sinsheimer, California Institute of Technology, Pasadena, California.

Solutions. 'Purified' CsCl was used as obtained from Fisher Scientific Co. (Fairlawn, New Jersey). The pH values of solutions were measured before use and adjusted to pH 7·4–7·8, usually with acid. The buffer used in the centrifugation was 0·5 M-tris (2-amino-2-hydroxy methyl propane-1:3-diol) adjusted to pH 7·2–7·6 with HCl.

Density measurement. The method of Jacobsen & Linderstrøm-Lang (1940) was used, with bromobenzene as the lower and a mixture of *m*-xylene and bromobenzene as the upper immiscible fluid. Even with water saturated columns, the slow drift of

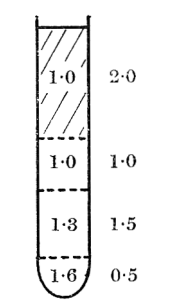
drops to higher densities was observed as reported by Miller & Gasek (1960). Readings were taken within 2 min. and calibrating drops were always used. Measurements were converted to d_4^{20}, the weight (g.) of 1 ml. of solution at 20°.

Preparative ultracentrifugation. A Spinco (Beckman Instruments, Spinco Division, Palo Alto, California) swinging bucket rotor SW-39 with cavity extending from 5·5 to 10·0 cm. was used in the Models E and L ultracentrifuges operated between 30,000 and 37,000 rev./min. at 4–15°, with most runs below 10°. In some preliminary equilibrium experiments, tube adaptors allowed the use of 2 ml. quartz or 0·8 ml. lusteroid tubes. The experiments started with the tube contents uniformly



Initial d_4^{20} (g./ml.)	Initial Vol. (ml.)	Final	Selected run Vol. (ml.)	Selected run d_4^{20} g./ml.	Selected run Recovery (%)	Summary Runs	Summary % Recovery Mean	Summary % Recovery s.e.
			1·0	1·380	2·7	8	1·4	± 0·5
			1·0	1·407	7·5	8	15·0	± 7·0
			1·0	1·436	40·8	9	19·0	± 9·0
			1·0	1·509	4·0	8	2·0	± 1·0
					55·0		37·4	

A



			3·6	< 1·1	1·7	13	0·3	± 0·3
			0·40	1·341	0·4	23	5·5	± 3·0
			0·55	1·417	88·5	25	40·0	± 12·0
			0·47	1·516	12·1	24	6·0	± 2·1
					102·7		51·8	

B

Fig. 1. Isodensity preparative ultracentrifugation of FMDV, type A by two methods: (A) field formed gradient; (B) non-linear preformed gradient. *Initial*-densities and volumes in SW39 tubes before centrifugation; shaded regions indicate infectious fluid. *Final*—appearance of tubes and volumes of fractions collected in selected runs after centrifugation; LS is light scattering zone. *Selected run*—density and % recovery of infectivity in each fraction: (A) 18 hr., 30,000 rev./min., 15°. (B) 4 hr., 37,000 rev./min, 6°. *Summary*—mean and standard error of the mean of the log percentage recovery averaged over the number of runs indicated.

distributed, having been adjusted to about 1·40 g./ml. density with CsCl. The non-linear isodensity experiments started with 2 ml. of infectious fluid layered over 3 ml. of a preformed gradient of CsCl as shown at the left in Fig. 1B. This initial gradient was made in the cold by layering first 0·5 ml. of 50 % (w/w) CsCl having

a density of 1·6 g./ml., then 1·5 ml. of 30 % (w/w) CsCl (a 1:1, v/v, dilution of the 50% (w/w) stock CsCl), and finally 1 ml. of buffer. The upper of these two boundaries was stirred by twirling a wire bent in a saw-tooth pattern to give a smooth gradient from 1·0 to 1·3 g./ml., whereas the lower boundary was left as a step from 1·3 to 1·6 g./ml., but it did not remain discontinuous because of diffusion and redistribution in the centrifugal field. In some experiments the volume of the starting sample was increased, which necessitated a decrease in the volumes of the upper two solvent layers forming the moving zone part of the centrifugation. The step from 0·5 ml. of the 1·3 g./ml. medium to 0·5 ml. of the 1·6 g./ml. medium was always retained.

After centrifugation, the tubes were removed, placed in a holder at 4°, and inspected by using a pocket flashlight. The light scattering zones could easily be seen in a darkened room looking roughly at right angles to the flashlight beam. Removal of samples was done successively from the top, primarily with a straight-tipped Pasteur pipette of 0·5–1·0 mm. bore. When only one fraction was desired, it was obtained by puncturing the tube and withdrawing with a syringe.

Calculations. The percentage recovery r_i of each fraction was computed from its volume and infectivity compared with the volume and titre of the starting material. The mean recovery $\bar{r}$ in a series of n replicates was calculated by averaging the log recovery values. Similarly, the error of the mean was determined from the standard deviation of the log recovery values ($\sigma_{\log r}$) as:

$$\text{error} = \bar{r}\left[\log^{-1}\left(\frac{\sigma_{\log r}}{\sqrt{n}}\right) - 1\right].$$

Computation on a log basis was chosen because the assay has a constant percentage error. This, however, gives a lower value for the mean than does averaging on a linear basis.

Optical ultracentrifugation. A 'Spinco' AN-E rotor with a 30 mm. cell was used at 50,740 rev./min. at 4° in the Model E ultracentrifuge. The sum of the deflexions of light caused by the concentration gradient set up by 40 % (w/w) CsCl at equilibrium and the compression gradient was so great under these conditions that three optical modifications were required: the first was to use quartz prisms as cell windows, $-1°$ as the lower and $-2°$ as the upper; the second was to move the schlieren light source off axis to the rear by about 1 cm., to augment the off-axis effect of the lower wedge window. This result could have been accomplished optically with two $-2°$ wedge windows, but would have precluded observation of the pattern early in the run, for which the light source had to be moved closer to the on-axis position. The compensation for the steep solvent gradients was necessary also in the absorption optical system, for which its light source was moved toward the front of the centrifuge. The third modification permitted freedom of movement of the phase-plate schlieren diaphragm across the optical track. This movement was essential to centre the pattern on the photographic plate at angles as low as 10° (Trautman, 1956).

Electron microscopy. Samples for electron microscopy were dialysed overnight against 0·2 M ammonium acetate (pH 7·6). After mixing with a convenient dilution of polystyrene latex particles (88 mμ diameter) containing bovine plasma albumin, or with 1 % (w/v) phosphotungstic acid neutralized with N-KOH the materials were sprayed from a modified nasal atomizer. Droplets were collected on copper

grids coated with an evaporated carbon film, shadowed with uranium when required, and examined in an EMU-3B microscope (Radio Corporation of America, Camden, New Jersey).

RESULTS

Fig. 1A shows the equilibrium method of isodensity zone centrifugation in which the initial density was adjusted to equal that of the particle of interest. Preliminary experiments were required to determine the proper density before the run illustrated was possible. Since a narrow light scattering zone was not seen with the low titre fluids used, relatively large volume fractions were taken. The density and recovered infectivity of each are given for a typical experiment. The density of the most infectious fraction was 1·436 g./ml. Nine such experiments are summarized in the right-hand column and show that the overall recovery was 37 %, with most in the third fraction.

Fig. 1B illustrates the preformed non-linear gradient method. In the selected experiment shown, 2 ml. of infectious concentrated tissue-culture fluid containing phenol red indicator were layered above the gradient. After centrifugation, the indicator extended from the meniscus through the debris, shown cross-hatched in Fig. 1B, while below this level the solution was transparent except for the narrow light scattering band indicated. The average density in the tube was only 1·15 g./ml., hence the virus would eventually form a pellet. The fraction containing the light scattering zone was about one-fourth the initial sample volume and contained 89 % of the initial infectious particles. The density gradient was steeper than in the equilibrium case of Fig. 1A.

Forty such experiments were performed with some differences in the volumes of the initial sample and in the fractions taken. When the initial sample differed from the standard 2 ml., the volumes of the 1·0 and 1·3 g./ml. layers were adjusted accordingly. Not all fractions were assayed in all experiments, nor were the titrations always successful. The average recoveries of those runs for which there were data are shown in the last column of Fig. 1B. The light scattering fraction is seen to have contained most of the infectivity with an average recovery of (40 ± 12) %. In these twenty-five experiments, the volume of this virus fraction varied from 1·3 to 20-fold. The mean value for 19 runs, which gave a concentration greater or equal to 5-fold, was 8·2-fold with a recovery of (47 ± 16) %, showing that the method can be used to concentrate virus solutions. The fractions immediately above and below the light-scattering fraction showed 1/7 of its infectivity. Hence it is reasonable to conclude that the infectivity coincides with the light-scattering material. In thirty-one experiments, in which the entire bottom fraction below the debris was removed, the average recovery was (75 ± 25) %.

To obtain the best value for the density of the infectious particle, the average was taken of all measurements on the most infectious fraction of the early equilibrium experiments and of those with the preformed gradient. There was a total of eighteen such measurements, giving a mean of $1{\cdot}430 \pm 0{\cdot}006$ g/ml.

Referring back to the last column of Fig. 1B, the over-all recovery in the tube was 52 %, with 0·3 % in the top 3 ml. In order to show that the latter was due to virus which had failed to sediment to the lower portion of the tube, a second centrifugation was performed, using the top fraction as the starting material; in two

such experiments the recoveries in the lower fraction were 59 and 70 %, with 6·2 and 3·7 %, respectively, remaining in the top fraction.

Plate 1, figs. 1 and 2, are electron micrographs of the virus fraction after the removal of CsCl. They show characteristic particles both by shadowing and in neutralized phosphotungstic acid (Bradish, Henderson & Kirkham, 1960). In 14 experiments in which the infectivity before and after dialysis was measured, the average recovery was (58 ± 32) % showing that small volumes (e.g. 0·3–1·0 ml.) could be dialysed without difficulty.

During these experiments, the infectivity was measured of 25 samples stored at 4° for periods ranging from 1 hr. to 20 days. The data show roughly that FMDV in 40 % (w/w) impure CsCl lost half its infectivity about every 4 days. This is to be compared to a half-life of 4 weeks of crude preparations without CsCl estimated from the data of Bachrach, Breese, Callis, Hess & Patty (1957).

The model systems of southern bean mosaic virus (SBMV, CsCl isodensity about 1·37) and bacteriophage ϕX-174 (CsCl isodensity about 1·40) were used in developing the preparative procedures (Sinsheimer, 1959). It was found that 50 μg. gave a readily distinguishable light-scattering zone, and that the gradient which developed in the region of the preformed step could resolve these two markers. A direct experiment in which 50 μg. of each marker was added to 2 ml. vesicular fluid revealed three light-scattering zones with the FMDV about twice as far below the ϕX-174 as the SBMV was above it. This indicated that the FMDV light-scattering material was about 1·46 g./ml., assuming the above isodensity values for the markers and making no allowance for non-linearity of the gradient. This suggests that the pipetting might have a slight systematic error tending to give too low a value for the density, perhaps by a drainage error. The entire bottom region of this tube was removed and centrifuged to equilibrium in the analytical ultracentrifuge. Plate 1, fig. 3, is a schlieren photograph of this run. Here the CsCl became redistributed to form the density gradient and the three viruses became separated in layers at their respective isodensity values. It should be noted that the biologically determined isodensity value allows identification of the right-hand large zone as, probably, infectious FMDV. The pattern also shows at about 1·5 g./ml. a smaller zone, marked with a question mark, which is specific to FMDV but which is presumably not the whole infectious virus.

DISCUSSION

The non-linear preformed gradient method described attempts to incorporate the advantages and reduce the disadvantages of the three major types of preparative ultracentrifugation. In particular, complete 'moving boundary' centrifugation (Trautman & Breese, 1959) concentrates the virus from a large volume into a pellet. It is inefficient, since pellets are difficult to resuspend, and are not depleted in materials of higher sedimentation coefficient than the one desired. Moreover, appreciable purification required many alternating cycles of low and high speed ('differential centrifugation'). The 'moving zone' type ('gradient differential', Anderson, 1955; 'rate zonal', Brakke, 1961) centrifuges virus from an initial zone to one between slower and faster contaminants. It thus does not require repeated cycles nor pellet resuspension. However, the initial zone can only be a fraction of the tube volume and a dilution rather than concentration is obtained in the virus

fraction. In neither of these two rate methods is there a separation from non-viral substances of similar sedimentation coefficient and, in both types, all the solutes will pack at the bottom, when centrifuged long enough. In contrast, the third type, 'isodensity centrifugation', gives a resolution based on particle density differences and generally can be made to give a concentration into a narrow final zone without a pellet to resuspend. Its two main subtypes depend upon whether the density gradient is preformed or 'field-formed' by the centrifugal redistribution of the small solute molecules of the 'solvent'. The field-formed type ('equilibrium density gradient'; Meselson *et al.* 1957) generally requires a long time to reach sedimentation equilibrium. The preformed type ('isopycnic gradient', Anderson, 1955; 'equilibrium zonal', Brakke, 1961) can be used for large particles, which will sediment or float to their isodensity positions before the field alters the gradient to its equilibrium value.

Various combinations of the fundamental types have been made either in successive centrifugations or in the same run. The combination used here achieves, in one tube, concentration and separation of the virus from contaminants of slower initial sedimentation coefficient of any particle density as well as separation from those initially faster but of different density. The use of the large step in density obviates any necessity for precise adjustment of initial densities. The 1–3 ml. initial volume is considerably more than the 0·5 ml. used in moving zone methods alone. When using 3–4 ml., the process is like the moving boundary method with a porous bottom that allows the virus to penetrate to its density of 1·4 g./ml., a density sufficiently greater than that of most of the nonviral contaminants which are thus concentrated above. The moving zone step for this agent, while not essential, should enable greater purity to be achieved in a shorter time than if the virus had to migrate the same distance always in the high density solution. For other systems in which the densities of the virus and the smaller debris are of the same order, the moving zone step would be required.

The increased rate of inactivation of FMDV, due to suspension in CsCl solution, of about 4 days half-life means that during an equilibrium run of 24 hr. 16 % would be inactivated, but in the 4 hr. run only 3% would be inactivated. Since the gradient is preformed, perhaps other salts in combination with D_2O might be found that would be less harmful. The time of the run has not been extensively studied, but it can be noted that a 4 hr. run at 37,000 rev./min in a 5 ml. SW 39 tube will deposit material of 24 S, which is about one-fourth the sedimentation coefficient of FMDV at 4° (Breese *et al.* 1960).

All the experiments in the present work cannot be considered replicates in the sense required for detailed statistical analysis. They represent all runs for which there was either a density or an assay measurement or both. The standard deviation on the log basis was about 0·6 for the various averages reported whereas the assay error itself is of the order of 0·3 log.

The excellent technical assistance of Mr C. E. Bennett in performing the infectivity assays is gratefully acknowledged.

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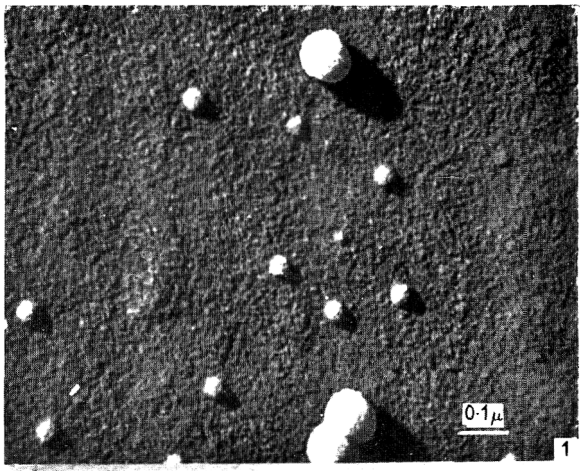
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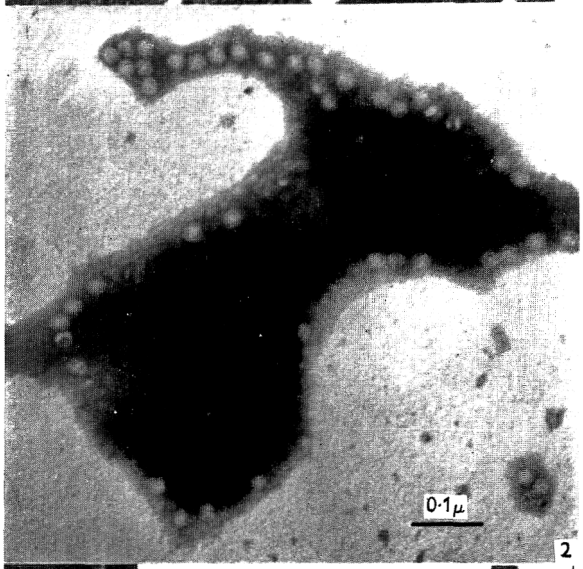
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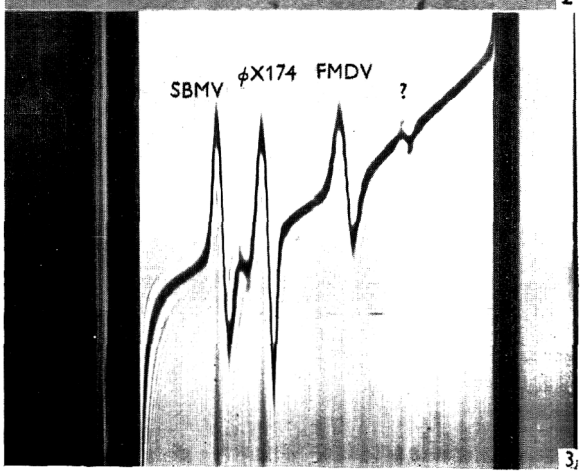
SBMV
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?
3

EXPLANATION OF PLATE

Figs. 1, 2. Portions of spray droplets showing FMDV, type A after dialysis of light scattering zone from isodensity ultracentrifugation: Fig. 1, shadowed with uranium, 88 mμ polystyrene latex markers. Fig. 2, negative staining with neutralized phosphotungstic acid.

Fig. 3. Isodensity sedimentation equilibrium a mixture of FMDV, type A and other viruses. Schlieren optical pattern after 704 min. at 50,740 rev./min. at 4°, phaseplate angle 60°. 1·0 ml. virus fraction from preformed gradient isodensity ultracentrifugation adjusted to 1·9 ml. and density 1·423 g./ml. Density increases to the right in the direction of the centrifugal field. The question mark is an unidentified zone.

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Growth of Elements of Various Sizes Found in Cultures of a Stable Proteus L Form

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(*Received* 27 *May* 1961)

SUMMARY

Samples of a liquid culture of Proteus L9 were transferred to agar blocks containing the same medium as the liquid culture. Photomicrographs were made of the slide cultures thus obtained, and the diameters of a large number of individual L elements determined. The measurements indicated that the elements of the L cultures studied could be assigned to two classes (I and II) with regard to their size. The elements of class I had an average diameter of 0·33 μ (standard deviation 0·071; standard error of the mean 0·006 μ); the corresponding figures for class II were 0·94, 0·21 and 0·02 μ, respectively. The growth of individual L elements in the slide cultures was followed during incubation at 30°. Only elements having a diameter $>$ 0·6 to 0·7 μ, i.e. elements belonging to class II, enlarged measurably or formed microcolonies. Calculations based on measurements made on L elements growing on streak plates confirmed these observations.

INTRODUCTION

Elements of various sizes are found in all cultures of bacterial L forms. Many investigators have noted in such cultures the occurrence of granular particles of a size approaching the resolving limit of the light microscope, in addition to elements of a size comparable to that of a normal bacterium. Whether the granular particles play a role in the life cycle of the L forms has been debated for several years (Kellenberger, Liebermeister & Bonifas, 1956; Tulasne, Minck, Kirn & Krembel, 1960; Klieneberger-Nobel, 1960; Weibull & Beckman, 1961). To elucidate this point some experiments with slide cultures observed by the phase contrast microscope (Knöll, 1944) have been performed by the present authors. A stable L form derived from *Proteus mirabilis* (Klieneberger-Nobel, 1956; Weibull & Beckman, 1961) was studied.

METHODS

Organism. The Proteus L form studied has been designated as strain L9 (Klieneberger-Nobel, 1956). It was derived from a strain of *Proteus mirabilis* (Weibull & Beckman, 1961).

Growth conditions. Samples of the L form were obtained for experimental purposes from stock cultures grown in 200 ml. Erlenmeyer flasks containing 50 ml. of the liquid serum-free medium described by Abrams (1955). No penicillin, however, was included in the medium used in the present work. The cultures were incubated at 30° for 24 hr. on a rotary shaker (100 rev./min.) and stored at room temperature.

Subcultures were made weekly. Bacterial material for slide cultures was obtained by growing Proteus L9 on Abrams's medium as described above for about 24 hr.

Preparation of slide cultures. Agar blocks approximately 1 mm. thick and containing 0·8 % (w/v) agar were used. The cultures were sealed with Vaspar (petroleum jelly). For precise location of individual L elements and colonies, Formvar films provided with lattice-like ruled areas were attached to the coverslips (Taubeneck, 1959).

Light microscopy. Photographs were taken using a ×90 Leitz phase contrast oil-immersion objective and a ×10 compensating eyepiece. Negatives obtained on Gevaert Graphic Ortho O 5 plates were enlarged twice when printed to give a final magnification of ×2600. The sizes of individual L elements were measured either on the photographic prints or under the microscope. In the latter case a ×25 eyepiece equipped with a micrometer was used. The distance between the rulings of the micrometer scale corresponded to 0·7 μ in the microscopic preparation when the oil immersion objective was used.

RESULTS

Preparation of medium for the slide cultures

Factors influencing the growth of Proteus L9 in pour plates and streak plates were studied by Weibull & Lundin (1961). No growth was obtained when fresh unsupplemented Abrams's medium (1955) was used for preparing the plates. When, however, 10 % (v/v) inactivated horse serum was added to Abrams's medium, viable counts of about 5×10^8/ml. were obtained in a 24 hr. Proteus L9 culture. An agar content of 0·7–0·8 % (w/v) was found to be optimal for growth.

A solid serum-free medium, giving only about 40 % lower viable counts in pour plates than the serum-containing medium described above, was prepared by centrifuging at 15,000 *g* an over-night culture of Proteus L9 grown in unsupplemented liquid Abrams's medium, killing the remaining viable L elements in the supernatant fluid by boiling or autoclaving this fluid, and adding 0·8 % (w/v) agar. This medium will subsequently be called 'old' Abrams's medium. It has now been found that the addition of 10 % (v/v) horse serum to 'old' Abrams's medium increases the viable counts three to four times. Consequently this serum-containing medium was chosen for the experiments described below. Some control experiments with fresh Abrams's medium supplemented with serum gave, however, essentially the same results.

The appearance of the slide cultures before incubation

Plate 1, fig. 1, shows the appearance of an uninoculated slide culture as viewed under the phase contrast microscope. The microscope was focused on the Formvar film which was attached to the lower surface of the coverslip and thus was in close contact with the agar surface. The dark lines represent replicas of the rulings in the glass matrix used for preparing the Formvar film (Taubeneck, 1959). The sides of the square are 25 μ apart.

Plate 1, fig. 2, and Pl. 2, fig. 4, show the appearance of a slide culture inoculated with Proteus L9 elements, previously grown in Abrams's liquid medium for about 24 hr. at 30°. It can be seen that most of the L elements were approximately spherical and had sizes varying from the resolving limit of the microscope to several μ.

Figure 1 shows the results of measurements performed on three slide cultures inoculated with different Proteus L9 cultures as described above. The measurements were made immediately after the inoculation. The diameters of all L elements (about 400) reproduced on three photographic prints were measured. It can be seen that the L elements can be assigned to two classes (I and II) according to their size. The average diameter of the particles belonging to class I is about $0{\cdot}3\,\mu$ and that of the particles of class II is about $0{\cdot}9\,\mu$. More than 97 % of the latter particles have a diameter $> 0{\cdot}6\,\mu$ and more than 99 % of the former ones have a diameter $< 0{\cdot}5\,\mu$. About 90 % of the particles of class I have a diameter $< 0{\cdot}4\,\mu$.

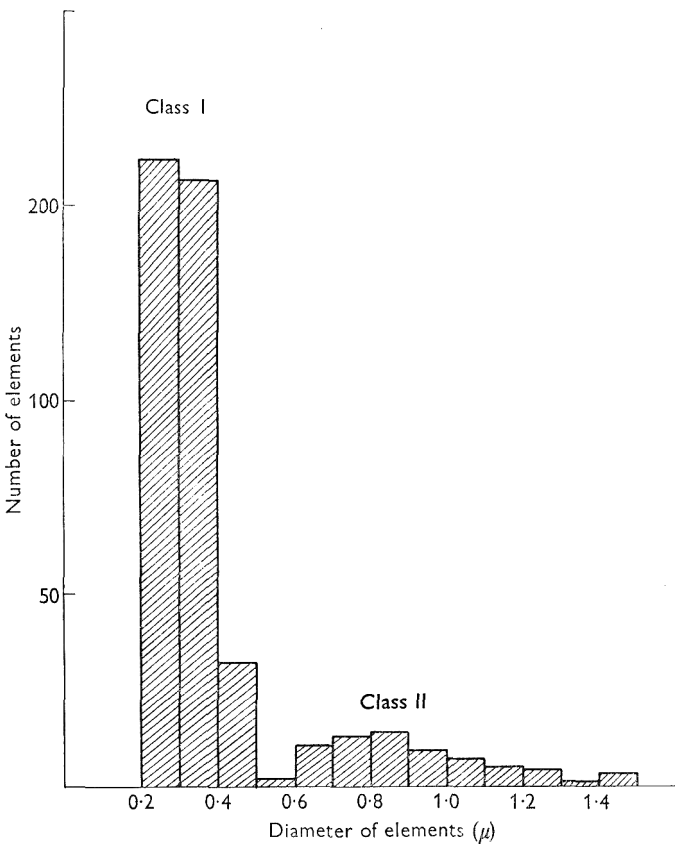

Fig. 1. Frequency of elements of various sizes in slide cultures of Proteus L9 before incubation. The L elements had previously been grown in Abrams's (1955) liquid medium.

The appearance of the slide cultures after incubation

Plate 1, fig. 3, and Pl. 2, fig. 5, show the appearance of two different slide cultures of Proteus L9 after incubation at 30° for 17 and 9 hr., respectively. Identical fields of view of these cultures before incubation are shown in Pl. 1, fig. 2, and Pl. 2, fig. 4. It can be seen that most of the larger L elements (diameter $> 0{\cdot}8\,\mu$) grew considerably during incubation. Some of these elements, however, lysed or remained unaltered in size. According to the photographs several of the small L elements (diameter $< 0{\cdot}6\,\mu$) were more or less displaced during the incubation of the slide

cultures. However, none of these elements with certainty increased their size as judged from the study of these photographs. Altogether, photomicrographs of slide cultures of eight different batches of Proteus L9 before and after incubation at 30° for 7–18 hr. were studied. The size of all particles exhibiting growth as judged by the unaided eye was determined. According to these measurements only particles of a diameter > 0·6–0·7 μ enlarged definitely during incubation. This value corresponds with the lower size limit of the L elements belonging to the particle class II in Fig. 1.

The average diameter of 102 L elements from four different batches of Proteus L9 and having a diameter > 0·6 μ was found to be 0·94 ± 0·02 μ (the ± sign indicates standard error of the mean) before incubation and 1·43 ± 0·05 μ after incubation for 7–17 hr. at 30° in slide cultures. The difference is statistically significant. Observations on about 200 L elements of a diameter > 0·6 μ showed that 76 % of these particles grew during incubation. The rest showed no increase in size or lysed.

The appearance of about 1000 L elements from eight batches of Proteus L9 and belonging to the class I of Fig. 1 (diameter < 0·6 μ) was studied before and after incubation at 30° for 7–18 hr. It never was observed with certainty that such an element grew to such an extent that after incubation it should be assigned to the class II of Fig. 1. Furthermore, the diameters of 150 sharply focused small L elements were measured on photographic prints representing unincubated slide cultures. The same measurements were made on about the same number of incubated elements. In this way, the average diameter of L elements belonging to the class I of Fig. 1 was found to be 0·33 ± 0·006 μ before incubation and 0·32 ± 0·008 μ after incubation. Thus the measurements performed did not indicate any growth of L elements of a diameter < 0·6 μ during incubation in the slide cultures.

Experiments with L cultures fractionated by means of differential centrifugation

The results given above agree with the findings of Weibull & Beckman (1961), obtained by means of chemical and radiochemical methods. According to these workers there is very little or no biosynthetic activity of small L elements (most of them having a diameter < 0·3 μ, as measured electron microscopically). To compare more closely the data obtained by means of the chemical methods with the results of the slide culture studies described in the present paper, L cultures were fractionated by means of differential centrifugation essentially as described by Weibull & Beckman (1961). A suspension of small L elements obtained by this procedure was suitably diluted in 'old' Abrams's medium and slide cultures of the suspension were prepared. As can be seen from Pl. 2, fig. 6, almost all of the microscopically observable elements are bodies near the resolving limit of the light microscope. Only occasionally larger elements were found (as indicated by the arrow in Pl. 2, fig. 6). The average diameter of about 100 sharply focused small bodies was found to be 0·35 ± 0·008 μ. After 18 hr. incubation the corresponding figures were 0·34 ± 0·009 μ, i.e. no measurable growth of these bodies could be observed. At the same time, however, contaminating L elements of larger sizes enlarged considerably and often formed microcolonies (Pl. 2, fig. 7). It should be pointed out that the size of the small elements in the fractionated L cultures (average value 0·35 μ) was practically the same as that of the small L elements found in unfractionated cultures (average value 0·33 μ).

Experiments with streak plates

In all the slide culture experiments described above, the L elements were incubated in close contact with ruled Formvar films which in turn were attached to the coverslips. This arrangement made it easy to find again after incubation any individual L element studied or photographed before incubation, even when the slide culture was removed from the microscope stage in the meantime. On the other hand, it could be argued that the growth of the small L elements might be inhibited more than that of the larger ones under the prevailing semi-anaerobic conditions. To test this possibility cultures of Proteus L9 were suitably diluted with 'old' Abrams's medium and spread as uniformly as possible on plates containing the same medium and 0·8 % (w/v) agar. The plates were incubated at 30° for about 24 hr. Agar blocks were removed from the plates before and after incubation and studied under the phase contrast microscope. Before incubation the number of L elements having a diameter of $\leqslant 0{\cdot}4$, 0·4–0·7 and $\geqslant 0{\cdot}7\mu$ was determined in several fields of view using the ocular micrometer. After the incubation, the number of microcolonies formed (average diameter about 20μ) was determined in the same way. Table 1 gives the results of these experiments. Some of the L cultures were fractionated by means of differential centrifugation.The fractionation was essentially carried out as described by Weibull & Beckman (1961).

Table 1. *Number of L elements of various sizes present in L cultures grown in Abrams's liquid medium and number of microcolonies formed after incubation of these cultures for about* 24 *hr. on streak plates containing 'old' Abrams's medium plus* 0·8 % *agar-agar and* 10 % *horse serum*

The L cultures used in two experiments (3 and 4) were fractionated by means of differential centrifugation according to Weibull & Beckman (1961). The diameter of the L elements was measured by means of an ocular micrometer. The ± sign indicates standard error of the average values given.

	No. of L elements per microscopic field of view and having a diameter of			No. of microcolonies after incubation
Expt.	$\leqslant 0{\cdot}4\mu$	0·4–0·7μ	$\geqslant 0{\cdot}7\mu$	
1	57·7 ± 3·8	1·56 ± 0·25	7·39 ± 0·67	4·61 ± 0·20
2	87·0 ± 8·6	4·90 ± 0·41	8·20 ± 0·53	5·50 ± 0·29
3	*c.* 350	0·79 ± 0·17	0·23 ± 0·06	0·19 ± 0·03
4	*c.* 500	0·37 ± 0·07	0·24 ± 0·08	0·40 ± 0·04

When unfractionated L cultures were studied (Table 1, Expts. 1 and 2) the number of microcolonies formed on the streak plates was about 30 % lower than the number of L elements of a diameter $\geqslant 0{\cdot}7\mu$ present on the plates before incubation. Experiments with slide cultures (see the preceding sections of this paper) indicated that about 75 % of similar L elements enlarged and that the rest remained unchanged or lysed during incubation at 30°. Thus the results of these two methods of investigation agree well. In any case the results of the streak plate experiments gave no evidence indicating that L elements having a diameter $< 0{\cdot}7\mu$ were able to grow.

The experiments with fractionated L cultures (Table 1, Expts. 3 and 4) gave essentially the same results as the experiments with whole L cultures, even if in

the former case the number of microcolonies was almost as high as (Expt. 2), or slightly higher than (Expt. 4) the number of L elements of a diameter $\geqslant 0{\cdot}7\mu$ present in these cultures before incubation. The total number of L elements of a diameter $> 0{\cdot}4\mu$, however, was still higher and that of the L elements having a diameter of $\leqslant 0{\cdot}4\mu$ was far higher.

DISCUSSION

Many attempts have been made to determine the size of the smallest viable elements present in cultures of bacterial L forms. Generally the filtration technique has been used. From results obtained by this technique, Kellenberger *et al.* (1956), Klieneberger-Nobel (1956), Tulasne & Lavillaureix (1958) and Rada (1959) concluded that the smallest viable elements of the stable Proteus L forms investigated had a diameter of about $0{\cdot}3\mu$. However, even when filtration experiments are carefully performed, the results obtained are not always easily interpretable. For example, soft, spherical particles may be forced through the filter pores in the form of threadlike elements. Such elements would have a considerably smaller diameter than the original spheres. A too-low value might thus be obtained for the smallest filterable elements of the L culture. Another source of error in a filtration experiment is due to the gradual plugging up of the filter pores. The number of elements of a certain size recovered in the filtrate may thus be considerably lower than that of the same elements in the original culture.

The technique of observing the L elements in slide cultures as described in the present paper eliminates the above-mentioned drawbacks of the filtration method. On the other hand the slide culture method implies that the L elements can be observed in the light microscope. Moreover, elements of a certain kind occurring at a very low frequency as compared to the total number of elements ($< 1:10^3$) may not be observed when the microscopic method is used.

The results obtained during the present investigation indicate, among other things, that individual elements of Proteus L9 of a diameter $< 0{\cdot}6\mu$ at the most very seldom grow. However, this finding does not necessarily contradict the results of the filtration experiments quoted above, since only between 1000 and 2000 L elements were studied in the present investigation and the result of the filtration experiments can be explained if only a few of about 10^5 particles having a diameter of $0{\cdot}3\mu$ are viable.

On the other hand, the slide culture experiments clearly indicate that the great majority of the small L elements (diameter $< 0{\cdot}6\mu$) do not grow under conditions that are very favourable for growth of the L culture as a whole. Therefore these elements probably do not play a major role in the ordinary life cycle of Proteus L9. The fact that the L elements with regard to their size can be assigned to two different classes, one of which contains only or almost only non-viable elements and the other mainly viable elements, is also noteworthy in this connexion.

Our results agree with the data recently published by Roux (1960). This worker studied an unstable L form of *Vibrio cholerae*, and stable and unstable L forms of Proteus P18. By using the micromanipulation technique Roux found that granular elements released from large L bodies never gave rise to colonies. Under the same cultural conditions up to 50 % of the large L bodies themselves were viable.

Weibull & Beckman (1961) showed that small L elements, most of them having

a diameter < 0·3μ as measured electron microscopically, exhibited a very low biosynthetic activity, if any. These elements were obtained by fractionation of whole L cultures by means of differential centrifugation. Essentially the same fractionation procedure was used in some of the present experiments. In agreement with the findings of Weibull & Beckman the isolated, small L elements did not grow in slide cultures. From measurements on photographs of the slide cultures the average diameter of these L elements was found to be 0·34 to 0·35μ. Since, however, relatively large systematic errors may be involved, especially in the light microscopic size determinations, essentially the same kind of fractionated L elements were very probably studied by the present authors and Weibull & Beckman (1961).

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EXPLANATION OF PLATES

The magnification in all figs. is ×2600.

PLATE 1

Fig. 1. Slide culture of Proteus L9 before inoculation as viewed under the phase contrast microscope. The microscope was focused on the Formvar film which was attached to the lower surface of the coverslip and thus was in close contact with the agar surface. The dark lines represent replicas of the rulings in the glass matrix used for preparing the Formvar film.

Fig. 2. Slide culture of L elements immediately after inoculation with a liquid culture of Proteus L9 previously grown in Abrams's liquid medium.

Fig. 3. Same culture as in Fig. 2 after 17 hr. incubation at 30°. Identical fields of view in Figs. 2 and 3.

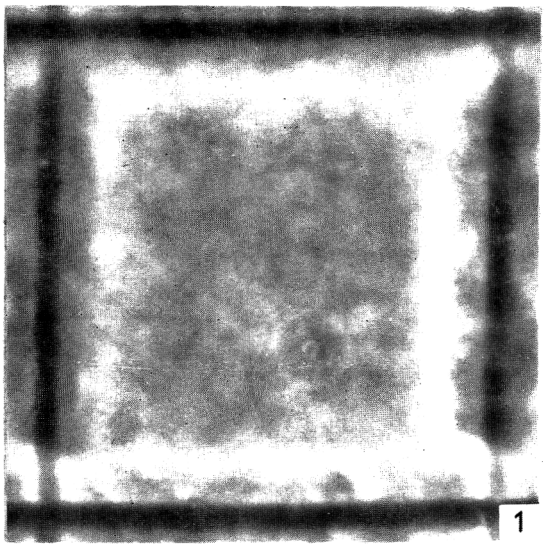
PLATE 2

Fig. 4. A slide culture of L elements other than that shown in Pl. 1 immediately after inoculation with a liquid culture of Proteus L9 previously grown in Abrams's liquid medium.

Fig. 5. Same culture as in Fig. 4 after 9 hr. incubation at 30°. Identical fields of view in Figs. 4 and 5.

Fig. 6. Slide culture of L elements immediately after inoculation with a liquid culture of Proteus L9 previously grown in Abrams's liquid medium. The culture had been fractionated according to Weibull & Beckman (1961).

Fig. 7. Same culture as in Fig. 6 after 18 hr. incubation at 30°. Identical fields of view in Figs. 6 and 7.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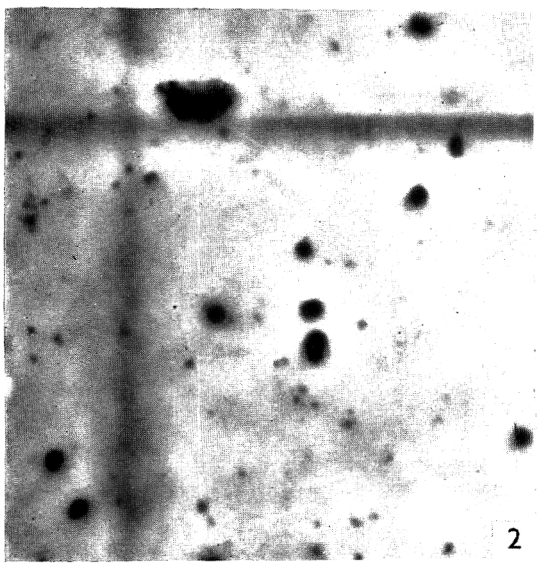
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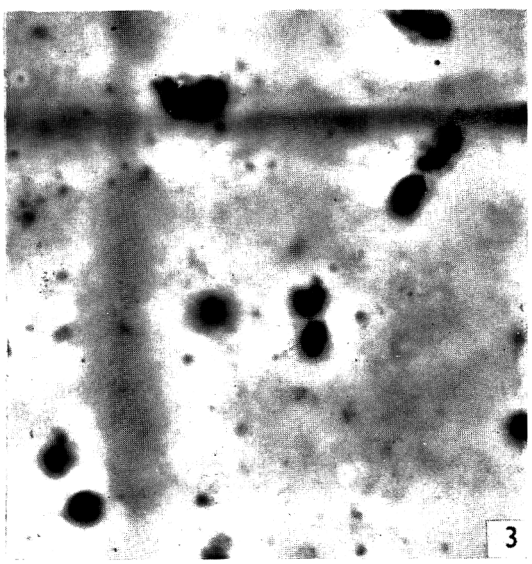
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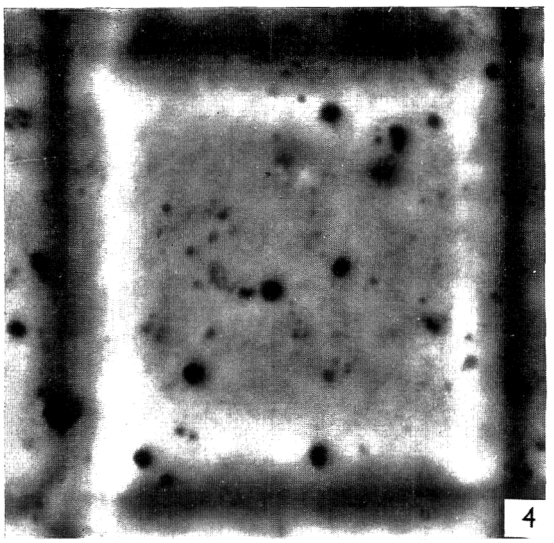
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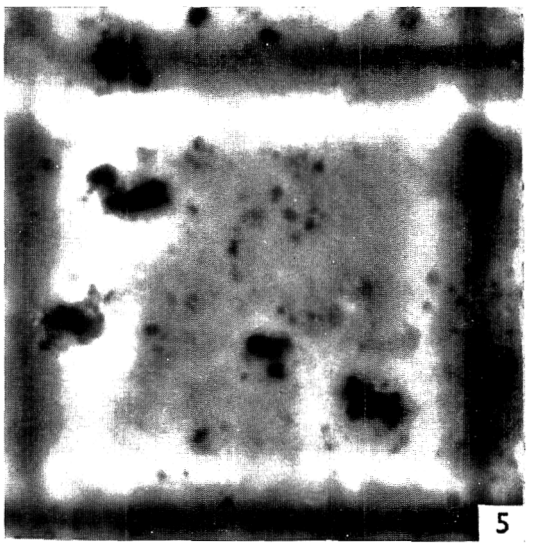
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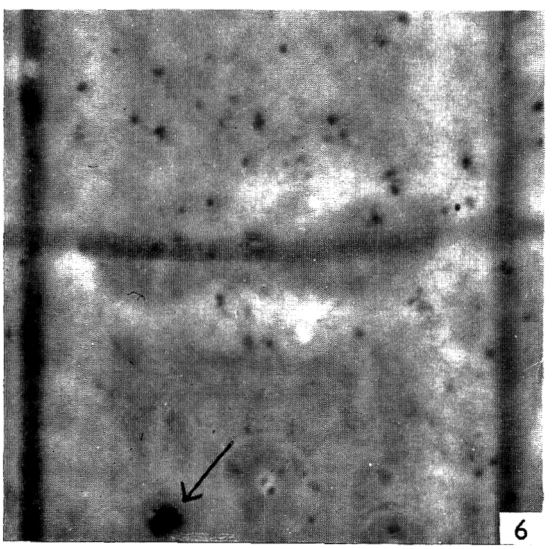
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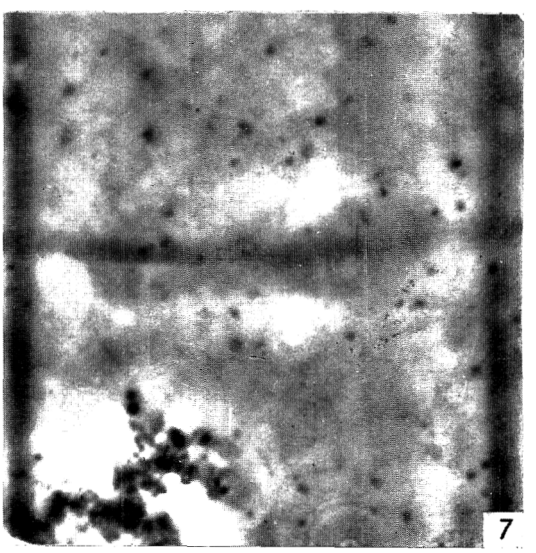
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The Antibiotic Relationships of Some Yeasts from Soil and Leaves

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SUMMARY

The antibiotic powers of some common soil and leaf bacteria, streptomyces and moulds were tested against soil and leaf yeasts. Twelve of the seventeen bacteria used, ten of the eleven streptomyces strains and seven of the twenty-two moulds were inhibitory. *Pseudomonas chlororaphis*, *Aeromonas* sp., five streptomyces and *Trichoderma viride* inhibited 70 % or more of the twenty-five yeast species used. The most frequently isolated soil yeasts were amongst those most sensitive to antibiotics. Only one species of leaf yeast but several soil yeasts were inhibited by leaf bacteria and moulds. As streptomyces do not occur on leaves, it seems that the wide seasonal fluctuations in kinds and numbers of yeasts on leaves are due rather to nutritional and physical factors than to antibiotic ones.

INTRODUCTION

In New Zealand it has been found that the soil yeast flora varies qualitatively from place to place with soil type and vegetation but not with season (di Menna, 1955, 1957, 1958, 1960*a*, *b*), whilst the yeast flora on the leaves of pasture plants changes in type with season but not with locality (di Menna, 1959*a*). Yeasts from pasture leaves reach the soil under them in large numbers but are unable to grow there or even to survive for long (di Menna, 1960*b*). An examination of some physiological characters of soil and leaf yeasts showed little difference between the two groups (di Menna, 1959*b*), so it was suggested that antagonistic factors originating from other members of the soil population kept the two floras distinct. The present work reports a study of some interactions between yeasts common in soil or on leaves and the bacteria, streptomyces and moulds which have been found to occur most frequently in soil and on pasture plant leaves in New Zealand.

ORGANISMS USED

The yeasts used as test organisms were much the same as those used in the examination of physiological characters (di Menna, 1959*b*). Some modifications of the list, deletions, additions and changes in probable habitat, have been made with progress in the survey work. Twenty-five species were used, eleven from soil, seven from leaves, and seven whose habitat is still uncertain but which have appeared sufficiently frequently in cultures from soil and leaves to warrant inclusion under the heading Uncertain Habitat. Two isolates of each species were used.

From soil: *Hansenula saturnus* (Klocker) H. & P. Sydow, *H. californica* (Lodder) Wickerham, *H. mrakii* Wickerham, *Cryptococcus albidus* (Saito) Skinner, *C. diffluens* (Zach) Lodder & Kreger-van Rij, *C. terreus* di Menna, *Candida humicola* (Daszewska) Diddens & Lodder, *C. curvata* (Diddens & Lodder) Lodder & Kreger-van Rij, *C. muscorum* di Menna, *Trichosporon pullulans* (Lindner) Diddens & Lodder, *T. cutaneum* (de Beurm., Gougerot & Vaucher) Ota.

From leaves: *Sporobolomyces roseus* Kluyver & van Neil, *Cryptococcus laurentii* (Kufferath) Skinner, *Torulopsis ingeniosa* di Menna, *Rhodotorula glutinis* (Fres.) R. Harrison, *R. mucilaginosa* (Jorg.) Harrison, *R. marina* Phaff, Mrak & Williams, *R. graminis* di Menna.

Uncertain habitat: *Saccharomyces delbrueckii* Lindner, *Debaryomyces kloeckeri* Guill. & Peju, *Cryptococcus luteolus* (Saito) Skinner, *Candida tropicalis* (Cast.) Berkhout, *C. guilliermondii* (Cast.) Langeron & Guerra, *C. parapsilosis* (Ashf.) Langeron & Talice, *Schizoblastosporion starkeyi-henricii* Ciferri.

One isolate of each of the antagonist organisms was used. Eleven strains of bacteria from soil and six from leaves were selected and provided by Dr J. D. Stout (see Stout, 1958, 1960*a*, *b*). The nomenclature is used according to *Bergey's Manual* (1957).

From soil: *Pseudomonas chlororaphis*, *P. fluorescens*, *Aeromonas* sp., *Achromobacter* sp., *Aerobacter cloacae*, *Serratia* sp., *Bacillus cereus* 'smooth' form, *B. cereus* 'rough' form, *B. sphaericus*, *Nocardia* sp., *Myxococcus* sp.

From leaves: *Xanthomonas* sp., *Flavobacterium* sp. 1, *Flavobacterium* sp. 2, *Aerobacter* sp., *Micrococcus* sp. 1, *Micrococcus* sp. 2.

Eleven strains of streptomyces from soil were used. Ten of these had been selected for their antibiotic producing ability and were provided by T. R. Vernon and one was provided by J. D. Stout. Three of these strains have subsequently proved to be of *Streptomyces hygroscopicus* and one of *S. pilosus*. Streptomyces are virtually absent from pasture plant leaves in New Zealand (T. R. Vernon, personal communication).

Fourteen moulds characteristic of soil and eight characteristic of leaves were selected and provided by R. H. Thornton (see Thornton, 1958, 1960).

From soil: *Absidia glauca* Hagem, *Mortierella alpina* Peyronel, *M. elongata* Linm, *Mucor hiemalis* Wehmer, *Zygorhynchus moelleri* Vuill, *Trichoderma viride* Pers. ex Fr., *Penicillium janthinellum* Biourge, *Gliocladium roseum* Bain, *Fusarium oxysporon* Schlecht emend. Snyder & Hansen, *F. culmorum* (W. G. Smith) Sacch., *Cylindrocarpon radicicola* Wr., *Papulospora* sp., *Rhizoctonia* sp., unidentified isolate SB 235.

From leaves: *Hormodendron cladosporoides* (Fr.) Sacc., *Fusarium avenaceum* (Fr.) Sacc., *F. nivale* (Fr.) Ces., *Pithomyces chartarum* (Berk. & Curt.) M. B. Ellis, *Stemphylium* sp. and unidentified isolates LA 190, LA 270 and HU 47.

In addition, the inhibitory effects of the following purified antibiotics were tested: gliotoxin, frequentin, patulin, viridin, mycophenolic acid and mixed *Gliocladium roseum* antibiotics (all supplied by Dr P. W. Brian, I.C.I. Laboratories, Welwyn, Hertfordshire, England), griseofulvin (Glaxo Laboratories, Greenford, Middlesex, England), actidione (Upjohn Co.) and polymyxin B sulphate (Pfizer Co.).

One medium was used, both for preliminary growth of the antagonists and for the growth of test organism with antagonist. This was the soy bean agar (SBA) of

Smith, Gordon & Clark (1952). All organisms used grew well on it, and although it is high in total nutrients it is low in reducing sugars (about 2·8 g./l.) which may interfere with antibiotic phenomena. The final pH value of the cultures varied between pH 6 and 8·5, but was usually on the alkaline side of neutrality.

A slightly different technique was used with each group of antagonists. Bacteria were streaked across the dried surfaces of SBA plates, four streaks per 10 cm. diam. plate. They were incubated for 4 days. Suspensions of yeasts in sterile tapwater were then spread between, but not touching, the bacterial colonies. There was no evidence that the preliminary bacterial growth had exhausted the nutrients in the medium.

Streptomyces were grown on SBA plates for 14 days. Plugs 0·6 cm. in diameter were cut from the cultures and placed, growth side uppermost, on SBA plates seeded with yeasts.

Moulds were grown on SBA plates for 7 days. Half the culture was then cut away along a diameter of each plate and replaced with sterile molten SBA. On this solidified surface yeast suspensions were streaked up to the cut edge of the mould culture.

Purified antibiotics were diluted to 1/1000. Sterile water was used as a diluent, except for some of the sparingly water-soluble compounds where the primary dilution of 1/100 was made up in a 1 + 4 by vol. acetone + water mixture. Sterile filter paper disks soaked in antibiotic solution were placed on the surface of SBA plates seeded with yeasts.

All cultures were incubated at 20°. Yeast cultures used as inocula were 2–7 days old. Yeast-antagonist cultures were read after 2 days of incubation and read again after 3 days of incubation.

RESULTS AND DISCUSSION

Results are given in Tables 1, 2 and 3 and are summarized in Table 4.

Twelve of the seventeen bacterial strains used showed inhibitory powers. *Aeromonas* sp. was most active, strongly inhibiting all the yeasts tested except *Trichosporon cutaneum* which it inhibited only weakly. *Pseudomonas chlororaphis* also inhibited all yeasts, but inhibition of *Candida muscorum* and *T. cutaneum* was weak. Only seven of the twenty-two moulds showed antibiotic powers. *Trichoderma viride* was most active; *Papulospora* sp. and *Cylindrocarpon radicicola* were the only others used in which more than a very low degree of activity could be demonstrated. Eight of the eleven streptomyces used inhibited more than half the yeasts tested.

Actidione, gliotoxin and viridin were the most strongly zymostatic of the purified antibiotics used. Polymyxin B, frequentin and mixed *Gliocladium roseum* antibiotics were moderately so. The antibiotic spectrum of viridin was similar to that of the isolate of *Trichoderma viride* used. At a concentration of 1/1000 the purified antibiotic inhibited both isolates of *Hansenula mrakii* which the growing culture used did not affect; the culture did not inhibit any yeasts resistant to the antibiotic. Mixed *Gliocladium roseum* antibiotics inhibited eleven yeast isolates belonging to eight species but the culture of *G. roseum* used inhibited none. Either the method employed was unfavourable to the production of *G. roseum* antibiotics, or else the strain used produced antibiotics at only a low concentration.

Cryptococcus laurentii, *C. albidus*, *C. luteolus*, *Candida curvata* and *Schizoblastosporion starkeyi-henricii* were the most sensitive of the yeasts examined, being inhibited by twenty or more of the fifty antagonist organisms used. The least

Table 1. *Sensitivity of soil and leaf yeasts to soil and leaf bacteria*

	Yeasts from soil											Yeasts from leaves							Uncertain habitat						
	T. cutaneum	*T. pullulans*	*C. muscorum*	*C. curvata*	*C. humicola*	*C. terreus*	*C. diffluens*	*C. albidus*	*H. mrakii*	*H. californica*	*H. saturnus*	*R. graminis*	*R. marina*	*R. mucilaginosa*	*R. glutinis*	*S. roseus*	*T. ingeniosa*	*C. laurentii*	*S. starkeyi-henricii*	*C. parapsilosis*	*C. guilliermondii*	*C. tropicalis*	*C. luteolus*	*D. kloeckeri*	*S. delbrueckii*
From soil																									
P. chlororaphis	±	+	±	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
P. fluorescens	·	·	·	+	·	+	·	+	·	±	+	+	·	·	·	·	·	·	·	·	·	±	±	·	+
Aeromonas sp.	±	+	+	+	+	+	+	+	·	+	+	+	+	+	+	·	·	+	+	+	+	+	+	·	+
Achromobacter sp.	·	·	·	+	·	±	·	+	·	·	·	·	·	·	·	·	+	·	·	·	·	·	·	·	+
Aerobacter cloacae	·	·	·	·	·	±	±	+	·	·	·	·	·	·	·	±	+	+	·	·	·	·	+	·	+
Serratia sp.	·	·	·	+	·	±	±	+	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	+
B. cereus 'S' form	·	±	·	·	·	·	·	±	±	·	·	·	+	·	·	±	·	+	+	·	·	·	·	·	+
B. sphaericus	·	·	·	+	·	+	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	+
Nocardia sp.	·	±	·	·	·	+	·	+	+	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	+
From leaves																									
Flavobacterium sp. 1	·	·	·	±	·	+	·	+	±	·	·	·	·	·	·	·	·	·	+	±	·	·	·	·	+
Aerobacter sp.	·	·	·	+	·	±	·	+	·	·	·	·	·	·	·	·	±	±	·	·	·	·	·	·	+
Micrococcus sp. 1	·	·	·	·	·	·	·	+	·	·	·	·	·	·	·	·	±	·	·	·	·	·	·	·	·

+, Inhibition of both yeast isolates by antagonist; ±, inhibition of one yeast isolate by antagonist, or weak inhibition of both.
No sensitivity was observed to *Bacillus cereus* 'rough' form or to *Myxococcus* sp. from soil, or to *Xanthomonas* sp., *Flavobacterium* sp. 2 or *Micrococcus* sp. 2 from leaves.

Table 2. *Sensitivity of soil and leaf yeasts to soil streptomyces*

	Yeasts from soil										Yeasts from leaves						Uncertain habitat				
	H. saturnus	*H. californica*	*H. mrakii*	*C. albidus*	*C. diffluens*	*C. terreus*	*C. humicola*	*C. curvata*	*C. muscorum*	*T. pullulans*	*T. cutaneum*	*C. laurentii*	*T. ingeniosa*	*S. roseus*	*R. glutinis*	*R. mucilaginosa*	*R. marina*	*R. graminis*	*S. delbrueckii*	*D. kloeckeri*	*C. luteolus*	*C. tropicalis*	*C. guilliermondii*	*C. parapsilosis*	*S. starkeyi-henricii*
×4.3	.	.	.	+	+	.	.	+	±	+	±	+	+	+	+	+	±	+	.	±	+	+	±	+	+
×4.4	+	+	.	+	+	+	.	+	+	+	.	±	+	+	+	+	+	+	.	±	+	+	+	+	+
×22.2	.	±	.	+	±	±	+	±	±	+	+	+	+	+	.	+	.	+	.	.	+	.	.	.	+
×37.2	±	.	.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	.	.	+	±	.	.	+
×59.2	+	+	.	+	+	+	+	+	+	+	.	±	+	+	±	+	±	+	.	±	+	.	.	.	+
×60.1	.	.	.	.	±	.	.	.	.	±	.	±	±	±	.	+	±	+	±	.	.	.	.	.	.
×62.1	.	.	.	±	.	.	±	+	.	+	.	.	.	.	+	.	+	.	.	.	+	.	.	.	.
×62.2	+	±	±	+	+	+	±	±	±	+	±	+	+	+	+	+	+	+	+	+	+	.	.	.	+
×62.3	+	+	+	+	+	+	+	+	+	+	+	+	±	+	+	+	+	+	+	+	+	.	.	.	+
70/7/1	+	+	.	+	.	.	.	.	.	+	.	.	±	+	.	.	.	+	.	.	+	.	.	.	+

+, Inhibition of both yeast isolates by antagonist; ±, inhibition of only one isolate.

One strain of streptomyces, ×11.1, showed no antibiotic powers. Strain ×4.3 has been identified as *Streptomyces pilosus*; strains ×37.2, ×62.2 and ×62.3 as *S. hygroscopicus*.

Table 3. *Sensitivities of soil and leaf yeasts to soil and leaf moulds and to some purified antibiotics*

	Yeasts from soil											Yeasts from leaves							Uncertain habitat						
	H. saturnus	*H. californica*	*H. mrakii*	*C. albidus*	*C. diffluens*	*C. terreus*	*C. humicola*	*C. curvata*	*C. muscorum*	*T. pullulans*	*T. cutaneum*	*C. laurentii*	*T. ingeniosa*	*S. roseus*	*R. glutinis*	*R. mucilaginosa*	*R. marina*	*R. graminis*	*S. delbrueckii*	*D. kloeckeri*	*C. luteolus*	*C. tropicalis*	*C. guilliermondii*	*C. parapsilosis*	*S. starkeyi-henricii*
Moulds from soil																									
C. radicicola	.	.	.	±	.	.	+	+	.	.	.	±	.	.	.	.	.	.	.	.	±	.	.	.	±
F. culmorum	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
F. oxysporon	.	.	.	.	.	.	.	.	.	.	.	±	.	.	.	.	.	.	.	.	.	.	.	.	.
Papulospora sp.	.	.	.	+	.	+	.	.	.	.	.	+	+	+	±	.	±	.	.	.	+	.	.	.	+
Rhizoctonia sp.	.	.	.	±	.	.	.	.	.	.	.	±	.	.	.	.	.	.	.	.	.	.	.	.	.
T. viride	+	+	.	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	+	+	.	.	.	+
Moulds from leaves																									
LA190	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Purified antibiotics																									
Actidione	+	+	+	+	+	+	.	±	+	+	.	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Frequentin	.	.	±	.	.	.	.	.	±	±	.	.	.	.	.	.	.	.	.	+	.	.	.	.	+
Mixed Gliocladium antibiotics	.	.	.	+	.	±	±	.	.	±	.	±	.	±	.	.	.	.	.	.	+	.	.	.	+
Gliotoxin	.	+	±	+	+	+	+	+	+	±	±	+	±	+	+	±	+	+	.	.	+	.	.	.	+
Viridin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	±	+	+	+	+	+	.	.	.	+
Polymyxin B	.	.	.	.	.	.	+	.	+	+	+	.	+	+	+	±	.	+	.	+	.	.	.	.	.

+, Inhibition of both yeast isolates by antagonist; ±, inhibition of only one isolate or weak inhibition of both.

No sensitivity was observed to *A. glauca*, *M. alpina*, *M. elongata*, *M. hiemalis*, *Z. moelleri*, *G. roseus*, *P. janthinellum* or unidentified SB235 from soil, to *F. avenaceum*, *F. nivale*, *H. cladosporoides*, *P. chartarum*, *Stemphylium* sp. or the unidentified LA270 and HU47 from leaves, or to the purified antibiotics patulin, mycophenolic acid or griseofulvin.

Table 4. *Total sensitivities of soil and leaf yeasts to bacteria, streptomyces and moulds from soil and leaves*

	Yeasts from soil											Yeasts from leaves							Uncertain habitat						
	H. saturnus	*H. californica*	*H. wrakii*	*C. albidus*	*C. diffluens*	*C. terreus*	*C. humicola*	*C. curvata*	*C. muscorum*	*T. pullulans*	*T. cutaneum*	*C. laurentii*	*T. ingeniosa*	*S. roseus*	*R. glutinis*	*R. mucilaginosa*	*R. marina*	*R. graminis*	*S. delbrueckii*	*D. kloeckeri*	*C. luteolus*	*C. tropicalis*	*C. guilliermondii*	*C. parapsilosis*	*S. starkeyi-henricii*
From soil																									
Bacteria (11)	3	3	5	8	4	7	2	7	2	3	2	5	3	3	2	2	3	3	9	4	6	3	2	2	6
Streptomyces (11)	6	6	2	9	8	6	6	8	7	10	5	8	9	9	7	8	8	8	3	4	9	6	2	4	9
Moulds (14)	1	1	0	4	1	2	2	3	1	1	1	5	2	2	2	1	2	1	1	1	3	0	0	0	3
Total soil strains	10	10	7	21	13	15	10	18	10	14	8	18	14	14	11	11	13	12	13	9	18	9	4	6	18
From leaves																									
Bacteria (6)	0	0	1	3	0	2	0	2	0	0	0	2	0	0	0	0	0	0	2	0	2	1	1	1	2
Moulds (8)	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Total all strains	10	10	8	24	13	17	11	20	10	14	8	20	14	14	11	11	13	12	15	9	20	10	5	7	20

Figures in table are numbers of organisms to which a yeast species has shown some sensitivity. Figures in parentheses are numbers of antagonist organisms used.

sensitive were *Hansenula saturnus*, *H. californica*, *H. mrakii*, *Debaryomyces kloeckeri*, *Candida tropicalis*, *C. guilliermondii*, *C. parapsilosis*, *C. muscorum* and *Trichosporon cutaneum* which were inhibited by ten or less. Some of these sensitivities are perhaps predictable in the light of what is known of yeast ecology, others seem paradoxical. Almost all the fermenting species tested (*Hansenula* spp., *S. delbrueckii*, *D. kloeckeri*, *C. tropicalis*, *C. guilliermondii* and *C. parapsilosis*) are included in the low sensitivity group. Fermenting yeasts generally appear to be commonest in habitats such as spoiling food, rotting fruit and fungus fruiting bodies, tree exudates and the animal gut, where nutrients are high, competition intense, and lack of sensitivity to antibiotics probably has high survival value.

Soil yeasts were relatively sensitive to antibiotics produced *in vitro* by other soil micro-organisms; indeed the most frequently isolated were the most sensitive. Of the soil yeasts with low sensitivities *Hansenula* spp. seem to be common in New Zealand only in those rarely occurring soils that are both water-logged and with a pH approaching neutrality (di Menna, 1960*b*; and unpublished). *Candida muscorum* has been found as the dominant yeast of only a few rather acid soils (Wright, 1959) where, according to the evidence of Jackson (1958), inhibitory effects will be decreased and resistance to antibiotics not a critical factor. *Trichosporon cutaneum* can be found in most New Zealand soils, but never as more than a small percentage of the total yeast population. It seems that resistance to antibiotics is not in itself of paramount importance in determining the ability of a yeast to live in soil.

It is perhaps significant that of the two rather similar soil species, *Candida humicola* and *C. curvata*, *C. humicola* was sensitive to eleven antagonist organisms and *C. curvata* to twenty. In New Zealand *C. curvata* has been the dominant yeast in a number of mineral soils and some acid peats. *C. humicola* on the other hand has been found mainly in the root zones of tussock plants (di Menna, 1958) and in the fermenting layer of forest litter (di Menna, 1960*b*), both relatively rich and presumably competitive environments.

Leaf yeasts as a group were almost completely insensitive to either leaf bacteria or moulds. The only demonstrable effects were that one isolate of *Cryptococcus laurentii* was inhibited by *Micrococcus* sp. 1 and that both *C. laurentii* isolates were weakly inhibited by *Aerobacter* sp. *Aerobacter* sp. and *Flavobacterium* sp. 1 showed moderate antibiotic activity against soil yeasts and yeasts of uncertain habitat, but of the group of eight leaf moulds the only one with any inhibitory powers was the unidentified LA 190 which inhibited *Candida humicola*. Although leaf bacteria may prevent some soil yeasts from establishing themselves in the phyllosphere, it seems that the great quantitative and qualitative changes which occur seasonally in the leaf yeast population are due to alterations in physical conditions and availability of nutrients rather than to antibiotic activities of the rest of the leaf microflora.

Leaf yeasts as a whole appeared only slightly more sensitive to soil bacteria, streptomyces and moulds than soil yeasts, so that it seems as though antibiotics play little part in preventing leaf species from establishing themselves in soil. However, the data obtained here show only the antibiotic producing potentialities of the antagonists tested, not the pattern of antibiotic activity in soil. The effective antibiotic producers in soil may be, for instance, *Papulospora* sp. and the streptomyces x4.3 and x60.1 which inhibit leaf rather than soil species. It will be necessary to work with soil itself to clarify this point.

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Growth of *Mycobacterium lepraemurium* Maintained in Cultures of Rat Fibroblasts

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SUMMARY

Hitherto only limited multiplication of *Mycobacterium lepraemurium* has been obtained in cell cultures; in cultures of rat fibroblasts (strain 14pf) used by Garbutt, Rees & Barr (1958) growth of bacilli was limited to one or two generations. The present work shows that more continuous intracellular growth of *M. lepraemurium* can be achieved in cultures of rat fibroblasts by repeatedly subculturing the infected cells. The results suggest that multiplication of the bacteria is maintained only when a high proportion (50–75 %) of the infected cells are transferred at each subculture. In one experiment, continued for 156 days, the increase in the number of bacteria was equivalent to 8 generations and the bacteria recovered from the cells were still infectious for mice. Quantitative electron microscopy also was used to follow the viability of bacteria from the cell cultures.

INTRODUCTION

Although rat and human leprosy bacteria have not been cultivated in bacteriological type media there have been several reports of significant though limited multiplication of the rat leprosy organism, *Mycobacterium lepraemurium*, in tissue cultures. Rees & Wong (1958) and Wallace, Elek & Hanks (1958) showed that *M. lepraemurium* multiplied in cultures of spleen tissue and cells, respectively, obtained from animals previously infected with rat leprosy. Rees & Wong (1958) showed that multiplication did not occur in otherwise identical cultures to which had been added high concentrations of antileprosy drugs (streptomycin, isoniazid). Primary cultures of explanted tissue or cells from previously infected animals seemed to offer the greatest chance of success because the bacteria would remain undisturbed in the specific host cells. On the other hand, such cultures could not be maintained in a healthy state for the long-term studies required for continuous multiplication of a slowly growing organism like *M. lepraemurium* which has a mean generation time of 10–13 days (Rees, 1957*b*). Such studies required the use of established lines of cells which could be infected *in vitro*. Wallace *et al.* (1958) obtained limited multiplication of *M. lepraemurium* in mouse fibroblasts (L strain) in the presence of high concentrations of hydrocortisone. Unfortunately the concentration of hydrocortisone required was damaging to the cells and the conditions therefore were not suitable for maintaining long-term cell cultures. Garbutt *et al.* (1958) obtained multiplication of rat leprosy bacteria in rat fibroblasts (strain 14pf) without the use of hydrocortisone. Multiplication in the majority of these cultures was confined to one generation, but in three experiments greater increases were

obtained (3·1-, 4·4- and 5·5-fold, respectively). No growth occurred in the cultures to which streptomycin and isoniazid had been added. In general, the initial rate of multiplication of the bacteria in all these tissue cultures corresponded closely to that obtained *in vivo* in the mouse or rat, but growth was limited to one or at most two generations, even when the tissue cultures were maintained for 40 days. In cultures of the primary type where tissues were obtained direct from animals with rat leprosy, the cells were deteriorating by the twelfth day. Even in the experiments with established cell lines, the cells deteriorated simply because the methods used were adopted to obtain accurate enumeration of the bacteria and did not necessarily provide the best conditions for maintaining healthy cells. For example, the medium was renewed infrequently, if at all, and the cells were not transferred. We report here more successful growth of *M. lepraemurium* in cultures of rat fibroblasts maintained by repeated subculture of cells infected *in vitro*.

MATERIALS

Cell cultures. A non-malignant line of rat fibroblast cells, strain 14pf (Gey, Hanks & Barrett, 1948; Ehrmann & Gey, 1953) was used throughout. Cultures of 14pf cells were received from Dr G. O. Gey in 1956 and 1958. The cell line had been maintained in medium containing human cord serum since it was first established in 1938, and all attempts in this laboratory to replace human cord serum by horse or calf serum were unsuccessful. Stock cultures of 14pf were maintained therefore in 50% (v/v) non-inactivated human cord serum (HCS 50) and 50% Hanks's balanced salt solution (BSS 50) with 100 units penicillin and 100 μg. streptomycin/ml. Cells were grown for 7 days, with twice-weekly changes of medium, in Porter flasks or in Pyrex feeding-bottles, and were subcultured by trypsinization (0·5%, w/v, trypsin, Difco 1:250).

Suspensions of Mycobacterium lepraemurium. The Douglas strain of *Mycobacterium lepraemurium* (Balfour-Jones, 1937) was used throughout. Liver from mice, albino P strain, infected intravenously 8–10 weeks previously with a partially purified suspension of bacteria (Rees, 1957*a*), was minced with scissors and ground in a mortar to a smooth suspension. All procedures were carried out under sterile conditions. To increase the susceptibility of the mice to *M. lepraemurium* the animals were given suramin (1 mg.) subcutaneously once weekly. The suspensions were usually prepared in 1% (w/v) albumin (bovine fraction V) in 0·85% (w/v) NaCl solution (albumin-saline). However, in some of the earlier experiments the suspensions were prepared in 0·5% (w/v) yeast supplement (Difco) and 5% albumin (w/v) in phosphate buffer (M/15, pH 7·4). The crude suspension was centrifuged at 1500 rev./min. (400 *g*) for 5 min. to remove tissue fragments and clumps of bacteria. The supernatant fluid was removed, centrifuged at 5000 rev./min. (5000 *g*) for 5 min. and the resulting supernatant fluid discarded.

The sediment was carefully resuspended in albumin saline and counted by the method described by Hart & Rees (1960). In most of these suspensions the bacteria were well dispersed and predominantly single organisms. All the procedures were carried out in the cold (2–4°). Immediately before adding to the cell cultures the stock suspension of *M. lepraemurium* was diluted in BSS or Parker's medium 199 (Morgan, Morton & Parker, 1950) to give a standard suspension containing

2–4 × 10 bacteria/ml. A fresh suspension was prepared for each experiment and used to infect the cell cultures within 4 hr. of removal of the tissue from the mouse.

Infection of cell cultures with Mycobacterium lepraemurium. For each experiment a bottle of 14pf cells was grown for 6–10 days to obtain a confluent sheet of cells. Four days before the experiment began 6 ml. of fresh medium was added to the bottle. Penicillin was decreased from 100 to 25 units/ml. and streptomycin omitted.

Before infecting the cells with *Mycobacterium lepraemurium* the medium was removed and the cells washed with BSS for 20 min. at 37° to remove excess serum which might be damaging to the bacteria (Hanks & Gray, 1954). The washed cells were then infected with *Mycobacterium lepraemurium* by adding 0·6 ml. bacterial suspension to 6 ml. 0·0125 % (w/v) albumin in BSS. Penicillin was added to a final concentration of 25 units/ml. and the cells incubated at 37° for 24 hr. During this period over 80 % of the cells ingested bacteria. After incubation the suspension of *M. lepraemurium* was removed from the bottle and the cells were recovered from the glass by adding 4 ml. trypsin and incubating for 10–15 min. at 37°. The trypsinized cells were thoroughly rinsed with BSS and lightly centrifuged to remove the extracellular bacteria.

Maintenance of cell cultures infected with Mycobacterium lepraemurium. The washed infected cells were resuspended in sufficient medium (HCS 50 BSS 50 + 25 units penicillin/ml.) to give a confluent sheet of cells in the appropriate containers (half test tubes, Porter flasks, or bottles) depending upon the design of the experiment. The cells were carefully maintained in suspension by agitation and were rapidly distributed, 1 ml. each to half test tubes or Porter flasks and 6 ml. to bottles. All infected cell cultures were incubated at 34° in order to slow the growth rate of the cells and to decrease the frequency at which the medium was changed. The cultures were examined every 2–3 days and the medium was changed every 10–12 days. Generally the cells were transferred by trypsinization every 21 days, the actual time being determined by the appearance of the cells. When the cells continued in very good condition transfers were delayed as long as possible, and by repeatedly subculturing 14pf cells infected with *M. lepraemurium*, healthy populations of cells were maintained up to 156 days.

Quantitative assessment of Mycobacterium lepraemurium *in cell cultures.* Quantitative methods were used to follow the total bacterial populations in the cell cultures by counting the acid-fast bacteria (Ziehl–Neelsen method) present in samples taken from the cultures at intervals through the experiment. The samples for counting were as follows: (*a*) base-line sample, taken at the beginning of the experiment or at the beginning of each subsequent transfer; (*b*) trypsinized sample, taken when the cells were trypsinized at the end of each culture period; (*c*) fluid-change sample, consisting of all the medium removed before adding fresh medium to the cultures. In order to follow the *total* bacterial populations in the cultures all volumes of cell suspensions, media removed at the time of renewal and samples taken for counting, were measured accurately. A standard procedure was used to prepare the samples for counting, but it was first necessary to decrease the high concentration of serum present, to prevent masking of the acid-fast bacteria in smears stained with carbol fuchsin. Measured samples (2–10 ml.) were therefore first centrifuged in sterile Lusteroid tubes at 5000 rev./min. (5000 *g*) for 30 min. To standardize the conditions of centrifugation, each sample where necessary was

made up to 10 ml. with sterile distilled water. As much as possible of the supernatant fluid was removed with a Pasteur pipette without disturbing the deposit and 1 ml. 0·1% (w/v) albumin in water added. To count accurately the total number of bacteria present in each culture it was first necessary to release completely the bacteria from the cells and so obtain a well-dispersed suspension of organisms. This was achieved in a single operation by exposing the tubes to ultrasonic vibration (300 kc./sec. for 30 sec.), a procedure which has been shown to disrupt the cells completely without damage to the intracellular bacteria (Wong, 1957). Immediately after exposure to ultrasonic vibration the sample was accurately re-measured, 0·05 ml. formaldehyde added, and the number of acid-fast bacteria counted by the method of Hart & Rees (1960). Standard spread smears were prepared from each sample and counted in quadruplicate. The spread smears were almost cell-free and the bacteria usually single. Whenever it was necessary to re-count a sample it was re-exposed to ultrasonic vibrations to disperse the bacteria.

Examination of Mycobacterium lepraemurium *in the electron microscope.* It has been shown that degenerate (dead) forms of *M. lepraemurium* can be identified in the electron microscope and that this technique is useful for following the survival time of these bacilli in tissue cultures (Rees, Valentine & Wong, 1960). In the present experiments selected samples of cultures for counting were also examined in the electron microscope for determining the proportion of dead organisms. To ensure that a sufficiently clean specimen was obtained for electron microscopy 10 ml. of sterile distilled water was added to each sample and centrifuged at 5000 rev./min. for 30 min. The supernatant fluid was removed leaving about 0·5 ml. and the deposit re-suspended by exposure to ultrasonic vibrations for 30 sec. The proportion of degenerate forms was determined, when possible, from 100 organisms.

Infectivity of Mycobacterium lepraemurium *recovered from tissue cultures.* Infectivity of the bacteria recovered from selected samples of cell cultures was determined in mice by the method of Rees *et al.* (1960). The samples were prepared by the same method as used for counting *M. lepraemurium*, but after the bacteria had been released from the cells, by exposure to ultrasonic vibrations, no formaldehyde was added. Five or 6 mice (albino P strain) were used for each sample tested. Standard doses were inoculated subcutaneously over the abdomen and the site inspected and palpated monthly for the presence of a leprosy nodule.

RESULTS

Altogether 20 cultures of 14pf cells were infected with *Mycobacterium lepraemurium*, but the cells in only 11 experiments were maintained successfully for more than one or two subcultures. Of the 11 experiments in which the cells were successfully subcultured, 8 were maintained by retaining 12·5–25% of the infected cells at each transfer (type A experiment) and 3 by retaining 40–80% of the cells (type B experiment).

Type A experiment

Thirteen cultures of this type were infected with *Mycobacterium lepraemurium*; 5 cultures were discarded early, 4 because of contamination and one because of poor initial uptake of bacteria. Of the 8 cultures which were successfully maintained

through several transfers, limited multiplication of bacteria was observed in 4 of them. The results of a representative experiment are now given in detail.

In the experiment the bottle of cells was infected with $1{\cdot}1 \times 10^9$ bacteria. After incubation overnight the cells were trypsinized and put into Porter flasks. Seven Porter flasks were used until day 152 and from then until the termination of the experiment (at day 195) the cells were maintained in a Pyrex feeding bottle. Fluid changes were carried out every 10 days and transfers at about every 20 days.

Table 1. *Percentage of uninfected and infected rat fibroblast cells (strain 14pf) showing the distribution of intracellular acid-fast bacteria (type A experiment)*

Culture period (days)	No. of bacteria/cell: 0	1–2	3–5	6–10	>10
	Infected cells (%)				
0	2	1	2	12	83
31	36	27	17	9	11
51	42	15	12	13	18
71	71	14	7	3	5
92	82	13	2	2	1
113	94	5	0	0	1
133	94	6	0	0	0
152	92	8	0	0	0
173	90	10	0	0	0
195	100	0	0	0	0

In an attempt to maintain the cells in the best possible condition by avoiding overcrowding in the flasks about one-quarter of the total cell population was used for each transfer. The distribution of bacteria in the cells was followed throughout the experiment by counting the acid-fast bacteria in stained smears prepared from a sample of cells taken at each transfer (Table 1). These direct smears showed a high initial uptake of bacteria by the cells (more than 90% infected) and also a steady decrease in the proportion of infected cells. Total bacterial counts were made at each transfer; the results are shown in Table 2. There were 4 culture periods in which there were about twofold increases in the number of bacteria. The total increase was 24·75-fold, or 4·5 generations in 152 days, giving an average generation time of 33·8 days. However, during 92 of the 152 days little or no multiplication of bacteria occurred; this accounts for the long generation time as compared with that found *in vivo*. When the generation time is calculated only on the 60 days in which significant multiplication occurred, then it would be 13·3 days. On days 51 and 71 the base-line samples were scored by electron microscopy for % degenerate (dead) organisms. When the number of degenerate organisms is subtracted from the total base-line count and from the subsequent trypsinized sample count (it was assumed that dead bacteria remained intact in the culture), then a greater apparent increase is obtained. On day 71 the apparent increase is 2·3-fold whereas, allowing for the degenerate bacteria, the estimated increase is 3·1-fold.

Viability of organisms recovered from cell cultures. On day 92 a suspension of bacteria recovered from the cell cultures was injected subcutaneously into 6 mice. The mice were palpated at regular intervals; all subsequently developed typical leprosy nodules.

Re-infected cell cultures. Six cultures of type A experiment were re-infected with a freshly prepared suspension of *Mycobacterium lepraemurium* at a time when only a few bacteria remained from the first infection. The cells were re-infected at periods ranging from 40 to 200 days after the primary infection. Five cultures were successfully maintained for a further one or more subcultures and in three a limited degree of multiplication of bacteria was observed. In a further four cultures an attempt was made to follow only the quality of the bacteria as determined in the electron microscope, throughout the experiment. However, this was not successful because the % of cells retained for transfer was too low; consequently there were insufficient bacteria for assessment.

Table 2. *'Apparent' and 'estimated' increase in the number of bacteria in cultures of rat fibroblast cells (strain 14pf) during each subculture (type A experiment)*

Age of culture (days)			Cells transferred (%)	No. of bacteria			
Total	Subculture	Sample		Total*	Degenerate by electron microscopy (%)	Increase† 'Apparent'	Increase† 'Estimated'
0	—	B.L.	—	2625	—	—	—
31	31	T.	—	3353	—	1·3	—
31	—	B.L.	25	452	—	—	—
51	20	T.	—	902	—	2·0	—
51	—	B.L.	25	145	39	—	—
71	20	T.	—	334	—	2·3	3·1
71	—	B.L.	25	42	11	—	—
92	21	T.	—	74	—	1·8	1·9
92	—	B.L.	25	16	—	—	—
113	21	T.	—	15	—	0	—
113	—	B.L.	25	6	—	—	—
133	20	T.	—	14	—	2·3	—
133	—	B.L.	25	1	—	—	—
152	19	T.	—	0	—	0	—

B.L. = base-line sample, T. = trypsinized sample, * = total number of acid-fast bacteria (X log 5·0755); † = 'Apparent' increase derived from (total count T/total count B.L.); 'estimated' increase derived from (total count T − no. degenerate bacilli in B.L.); (total count B.L. − no. degenerate bacilli in B.L.).

Type B experiments

Seven cultures of this type were infected with *Mycobacterium lepraemurium*; four cultures were discarded early, three because of contamination and one because of poor initial uptake of bacteria. Three cultures were successfully maintained through several transfers and in two of them multiplication of bacteria was also maintained. The results of a representative experiment are now given in detail.

The bottle of cells was infected with $2{\cdot}4 \times 10^9$ bacteria and the following day the trypsinized cells were transferred to half test tubes. The object in this experiment was to expand the cultures gradually from small, through intermediate, to large containers as the number of cells increased, in this way keeping the cells in good condition but metabolizing slowly. Half test tubes were used for the first 17 days,

Porter flasks to day 48 and Pyrex feeding bottles until the termination of the experiment on day 156. A much higher proportion (40–80 %) of cells was retained for transfer than in the type A experiments (Table 4).

The distribution of bacteria in the cells was again followed by counting the acid-fast bacteria in stained smears prepared at each transfer (Table 3). The initial uptake of bacteria was high, 84 % of the cells being infected. The decrease in the proportion of infected cells throughout this experiment was much less than in the type A experiments, and even from days 83 to 156 more than 50 % of the cells contained bacteria.

Total acid-fast bacterial counts and the proportion of degenerate bacteria, seen in the electron microscope, are shown in Table 4. During the total culture period the 'apparent' increase in the number of bacteria was 59·4-fold or 5·9 generations, which gives an average generation time of 26·4 days. Allowing for the proportion

Table 3. *Percentage of uninfected and infected rat fibroblast cells (strain 14pf) showing the distribution of intracellular acid-fast bacteria (type B experiment)*

Culture period (days)	No. of bacteria/cell: 0	1–2	3–5	6–10	>10
	Infected cells (%)				
0	16	10	8	6	60
17	8	10	12	18	52
48	18	14	26	16	26
83	49	19	8	9	15
106	31	22	19	9	19
156	41	21	19	10	9

Table 4. *'Apparent' and 'estimated' increase in the number of bacteria in cultures of rat fibroblast cells (strain 14pf) during each subculture (type B experiment)*

Age of culture (days)			Cells transferred	No. of bacteria			
					Degenerate by electron microscopy	Increase†	
Total	Subculture	Sample	(%)	Total*	(%)	'Apparent'	'Estimated'
17	—	B.L.	79	1448	19	—	—
48	31	T.	—	1532	—	1·06	1·07
48	—	B.L.	66	254	59	—	—
83	35	T. (1, 2)	—	912	—	3·59	7·32
	35	T. (3, 4)	—	997	—	3·92 (3·76)	8·14 (7·73)
83	—	B.L.	40	267	73	—	—
106	23	T. (1, 2)	—	371	—	1·38	2·44
	23	T. (3, 4)	—	393	—	1·47 (1·43)	2·75 (2·6)
106	—	B.L.	40	35	40	—	—
156	50	T. (2)	—	490	—	14·0	22·66
	50	T. (3)	—	307	—	8·77 (10·52)	19·45 (16·87)
	50	T. (4)	—	308	—	8·8	14·0

B.L. = base-line sample; T. = trypsinized sample, the bracketed number indicates individual bottles, pooled or treated singly; * = total number of acid-fast bacilli (X log 5·0755); † = 'Apparent' increase derived from (total count T./total count B.L.); 'estimated' increase derived from (total count T. − no. degenerate bacilli in B.L.); (total count B.L. − no. degenerate bacilli in B.L.).

of degenerate bacteria in each sample, as determined in the electron microscope, the increase was greater—362·7-fold or 8·4 generations—which gives an average generation time of 18·6 days. The 'apparent' and 'estimated' increase in the bacterial population during each subculture are shown in Fig. 1.

Viability of Mycobacterium lepraemurium *recovered from cell cultures.* On days 83, 106 and 156 samples of bacteria from the cultures were inoculated subcutaneously into mice and all the animals subsequently developed typical skin lepromata, showing that the bacteria were still viable and had retained their infectivity.

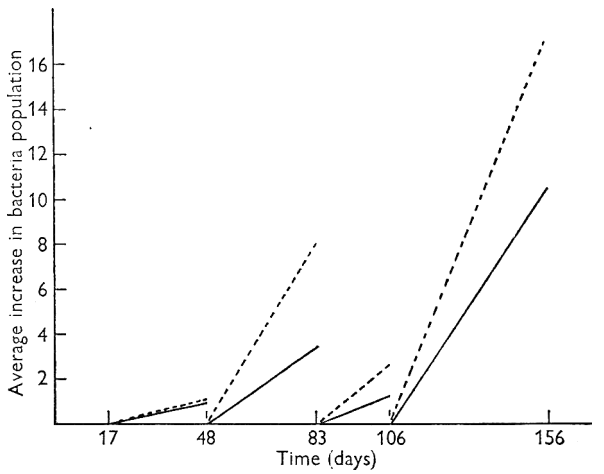

Fig. 1. 'Apparent' and 'Estimated' increase in the number of bacteria in rat fibroblast cells (strain 14pf) during each subculture (type B experiment). —— 'Apparent' increase; – – – 'estimated' increase.

DISCUSSION

This study shows, for the first time, that more continuous multiplication than hitherto of *Mycobacterium lepraemurium* can be achieved *in vitro* in tissue cultures. For example, the increase in the number of bacteria in several experiments was equivalent to 3 or 4 generations, and in one of the more successful experiments, the increase represented more than 8 generations. Hitherto, in our own work (Garbutt *et al.* 1958) and that of Wallace *et al.* (1958), multiplication of *M. lepraemurium* was generally confined to one generation. In the previous studies the cell cultures infected with mycobacteria were maintained for 2–6 weeks without subcultivation, whereas in the present work the infected cells were maintained for very much longer periods (up to 22 weeks) by repeatedly and regularly subculturing the infected cells. Since rat fibroblasts grow relatively slowly, particularly at 34°, new medium was required only every 10 days, but the cells were subcultured as soon as they showed deterioration, usually after 20 to 30 days. Changes in the bacterial populations were determined in each subculture period throughout the experiments. Although the methods were less accurate than those used in the earlier studies because of the necessity to transfer the cells from one container to another, they were sufficiently accurate to detect any significant multiplication. The individual culture periods were not less than 20 days; long enough to detect multiplication

if the bacteria were dividing freely at the average dividing time of 10–13 days found *in vivo*. The results of one particular experiment show that under these conditions the bacteria can continue to multiply in subcultured cells for 156 days, and this experiment was terminated only because of contamination. Nevertheless, multiplication did not occur in all the experiments and even in the successful ones the bacteria did not multiply regularly in each subculture.

The host cells chosen for these studies, rat fibroblasts (strain 14pf), were the same as those in which we had initiated successfully the growth of rat leprosy bacteria. The cell type used for growing rat leprosy bacteria may be important because, with identical methods of assessment, multiplication did not occur in these laboratories in mouse monocytes, in mouse fibroblasts (L-strain), with or without hydrocortisone or in monkey kidney cells. Nevertheless, it may be significant that growth of rat leprosy bacteria has been achieved only in cells derived from animal species susceptible to the infection. The rat fibroblasts are useful for long-term cultures because they can be adapted to grow slowly. Furthermore, they readily phagocytose rat leprosy bacteria and, under suitable conditions, 80–90 % of the cells can be infected. Normally fibroblasts will phagocytose the mycobacteria only in the presence of serum, but because serum has been shown to decrease the endogenous metabolism of *Mycobacterium lepraemurium* (Hanks & Gray, 1954) methods were developed (E. W. Garbutt; unpublished) to avoid direct exposure of the bacteria to serum. It was found that *M. lepraemurium* suspended in low concentrations of albumin were ingested by rat fibroblasts as readily as bacteria in a serum-containing substrate.

Rees *et al.* (1960) showed that electron microscopy can provide a quantitative guide to the viability of *Mycobacterium lepraemurium* by allowing dead forms to be identified. The method has been of great value in following the survival of bacteria in the tissue cultures, because rat leprosy bacteria, like other mycobacteria, still retain their ability to stain with carbol-fuchsin even when dead.

In earlier studies (Rees & Wong, 1958; Garbutt *et al.* 1958) it was stressed that multiplication of *Mycobacterium lepraemurium* could be determined accurately only by counting the total number of stained bacteria in each culture; other methods based on the proportion of infected cells or the distribution of bacteria within cells would be inaccurate because of the changing cell populations. This point is particularly well demonstrated in Tables 1 and 3, showing, in 2 experiments, the distribution of bacteria in samples of cells taken at each subculture. In these experiments the rat fibroblasts were in good condition and were multiplying freely. However all the cells, infected or uninfected, were not necessarily dividing at the same rates. From days 106 to 156 (Table 3) the proportion of uninfected cells remained fairly steady and the proportion of heavily infected cells decreased, yet there was, in fact, an increase in the total number of bacteria. Furthermore, the results in Table 1 showed a continuous increase in the proportion of uninfected cells, yet again they did not show that, from time to time, the total number of bacteria in the cultures increased. The results indicated, however, that the rate of bacterial multiplication was much less than the rate of cell division.

The present studies show quite definitely that continuous multiplication can occur in infected cultures which are regularly subcultured, as depicted in the type B experiments. In the type A experiments, although some multiplication was

obtained in several of the subcultures, it was not maintained regularly, and furthermore the number of bacteria decreased throughout the culture period. The type A experiments, in fact, illustrate the problems arising when attempts are made to culture an intracellular parasite with a much longer division time than the host cell. While it is necessary to subculture in order to maintain a healthy population of host cells, the proportion of cells transferred at each subculture must be high enough to retain sufficient bacteria for counting. In the type A experiments approximately three-quarters of the cells were discarded at each transfer in order to avoid overcrowding of the cells, but it is obvious now that this was too high a proportion to leave sufficient bacteria for accurate counting, even when some multiplication of bacteria occurred. In the type B experiments, only a quarter of the cells were discarded at each transfer and even though this resulted in overcrowding of the cells the cultures could be maintained for 20 to 30 days and the problem of insufficient bacteria for accurate counting did not arise.

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The Rate of Growth of *Salmonella typhimurium* with Proline or Glutamate as Sole C Source

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SUMMARY

Salmonella typhimurium will grow in simple defined media containing proline or glutamate as sole carbon source; in these media the organisms grew at 0·7 and 0·9 doublings/hr., respectively. Since most of the proline fed to the organisms is converted endogenously to cell material via glutamate, an attempt was made to see what reaction limited growth when the organisms were growing in proline as sole carbon source. The results suggest that the step which controls the conversion of proline to glutamate endogenously is the rate-limiting step. The uptake of proline from the medium to the free amino-acid pool did not appear to be rate limiting under these conditions. There was no evidence that the need to synthesize additional enzyme proteins (the proline degradative pathway) was responsible for the decreased growth rate of organisms growing on proline alone.

INTRODUCTION

The growth rate of bacteria is known to vary widely depending on the composition of the growth medium. Thus, by the use of appropriate media balanced growth of *Salmonella typhimurium* has been studied at rates anywhere from 0·6 to 2·8 doublings/hr. (Schaechter, Maaløe & Kjeldgaard, 1958). No attempt was made to determine which metabolic reactions were responsible for the observed differences in growth rate. We have now tried to identify such reactions by comparing the growth of *S. typhimurium* in simple defined media containing L-glutamic acid or L-proline as sole carbon source. These compounds were chosen since the only known pathway whereby proline is degraded endogenously to supply carbon for cell growth involves conversion to glutamic acid. As the growth rate on glutamic acid is higher than that on proline, there seemed to be some chance of locating the rate-limiting reaction in the proline culture. Preliminary experiments with *S. typhimurium* showed that, as expected, most of the carbon from proline flowed to the cell by way of endogenous glutamic acid. The slower growth on proline could therefore be due to one or more of the following causes: (1) the rate of flow of proline into organisms might limit the supply of carbon; (2) the rate of conversion of proline to glutamic acid within the cell could be limiting; (3) the induced enzymes which convert proline to glutamic acid might constitute such a large proportion of the total cell protein that other

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essential enzyme systems would be relatively reduced. The only well-authenticated case for the production of an enzyme decreasing the growth rate of an organism was provided by Novick & Weiner (1957) who showed that 'gratuitous' induction of β-galactosidase in *Escherichia coli* significantly increased the doubling time of cultures growing in a chemostat. The experiments described below favour the conclusion that conversion of proline to glutamic acid is the rate-limiting reaction in organisms growing in a simple defined medium with L-proline as the sole carbon source.

METHODS

Organisms and growth media. Salmonella typhimurium (the strain used by Schaechter *et al.* 1958) was maintained routinely by subculture every 2 or 3 weeks on minimal medium agar containing 2 mg. L-proline/ml. The slopes were incubated for 48 hr. at 37° and then stored at 2° until required for use. The purity and identity of the cultures were checked at intervals by plating out and by slide agglutination with specific sera prepared against the parent strain. Experiments in liquid media were carried out in medium 63 to which different concentrations of L-proline or L-glutamic acid were added before use. The medium 63 contains: KH_2PO_4, 0·1 M; NH_4Cl, 0·02 M; $MgSO_4$, 10^{-3} M; $FeCl_3$, 10^{-5} M; KOH to pH 7·0 ± 0·1.

Amino acids. L-proline and L-glutamic acid (California Corporation for Biochemical Research, Grade A) were used throughout. The L-glutamic acid solutions were pre-adjusted to pH 7·0 with 0·1 N-NaOH. The L- (*generally labelled*) -(^{14}C)-proline was obtained from the Radiochemical Centre, Amersham, Buckinghamshire, England. It was found to contain about 5–6 % of radiochemical impurity and was purified by two-dimensional chromatography in *n*-butanol + acetic acid + water followed by phenol + ammonia (see later).

Membrane filters. Filters with pore diameter about 400 mμ were obtained from the Membranfilter-Gesellschaft, Göttingen, Germany. These filters were found to contain large amounts of glycerol added as a plasticizer during manufacture. Consequently cultures constituted with filtered organisms always contained glycerol. Control experiments were carried out with filters from which glycerol had been removed by boiling in three changes of distilled water. These experiments showed that the results to be presented in this paper were not affected by the small quantities of glycerol introduced when organisms were shifted to a new medium after first being collected and washed on a filter.

Preparation of cultures. Medium containing 1 mg. proline/ml. was inoculated from an agar slope and the culture shaken overnight at 37°. In the morning the organisms were collected on a membrane filter and resuspended to an optical density of about 0·05 (Zeiss spectrophotometer; see later) in fresh medium. This culture was incubated at 37° for at least 2·5 generations to ensure that exponential growth at the characteristic rate had been established. The organisms were then collected and washed on a filter and resuspended in the specified media.

Balanced growth at low proline concentrations. This condition was achieved as follows. A given volume of medium was inoculated with exponentially growing organisms to an optical density of about 0·05. After incubating the culture with aeration for 15 min. (about 0·2 generation time) a portion was removed which decreased the number of bacteria to that contained in the original inoculum. The

culture was then restored to its initial volume by the addition of fresh medium containing sufficient proline to replace that used by the organisms and lost on removal of medium. This process was repeated 5 times. In a typical case 2000 ml. medium 63 containing 10 μg. proline/ml. was inoculated and after 15 min. 260 ml. of culture were removed and replaced by an equal volume of fresh medium containing 21 μg. proline/ml. In this way the optical density of the culture was kept between 0·05 and 0·062 and the proline concentration between 10 and 8·6 μg./ml.

Measurement of amino acid 'pools'. A carefully measured volume (500 ml.) of a culture of known optical density (between 0·05 and 0·07) was filtered rapidly (< 2 min.) through a 9 cm. membrane filter and washed with six lots of 20 ml. each of medium 63 at 2°. The membrane was then carefully removed from the filter assembly, washed on the underside with cold medium from a wash bottle to remove any contaminant that might have splashed up from beneath when the vacuum was broken in the flask, and submerged in 6·0 ml. of 5 % (w/v) trichloroacetic acid (TCA) aqueous solution at 2° in a Petri dish. After 15 min. the TCA solution was decanted and replaced by 4 ml. fresh cold TCA solution. After a further 15 min. the second batch of TCA solution was decanted off, the membrane removed and the contents of the Petri dish washed out with 2 portions of 1·5 ml. 5 % (w/v) TCA solution. All extracts and washings were combined. This procedure liberated the 'pool' components of the cells but also dislodged some of the bacteria from the membrane. The 'pool' compounds were separated from the bacteria by filtering through a 2·9 cm. membrane filter and washing the filter twice with 1·0 ml. of 5 % (w/v) TCA solution. The total volume of filtrate obtained in this way was between 14·0 and 14·5 ml. To show that all the proline contained in such preparations had in fact been extracted from the cells the following control experiment was carried out. A filter, through which 500 ml. of culture grown in medium 63 containing 10 μg. proline/ml. had passed, was washed with 50 ml. of medium 63 containing 2 mg. proline (i.e. 400 μg./ml.) and then with six 20 ml. portions of medium 63. The pool concentration determined in this experiment was about 1·5 times the concentration characteristic of organisms grown in 10 μg. proline/ml. It is therefore unlikely that pool concentrations measured on cultures grown in media with 2 mg. or less proline/ml. were due to non-specific adsorption on the organisms or on the filters.

Proline was determined directly on 5 ml. samples of the extract by the method of Troll & Lindsley (1955). When both proline and glutamic acid were to be determined, the extract itself was first treated with ether three times to remove the majority of the TCA and then desalted on 4·5 ml. columns of Dowex 1 (40–60 mesh; $\times 8$; H^+ form). The eluates were bubbled with air at 37° for 20 min. to remove ammonia and then taken to dryness at 100° in a stream of air. Glutamic acid and proline were separated by two dimensional chromatography and estimated on the paper after spraying the appropriate areas with ninhydrin (Mandelstam, 1958) and with isatin, respectively (Acher, Fromageot & Jutisz, 1950). The proline figures obtained in this way were checked by direct estimation of proline on representative samples removed before and after the desalting procedure.

Preparation of protein+cell wall hydrolysates. Samples containing about 1 mg. dry wt. of organism were fractionated to yield the protein+cell-wall fraction as

described by Richmond (1960). This material was hydrolysed in 6N-HCl for 16 hr. at 105° in sealed tubes. The HCl was evaporated at 100° in a stream of air, remaining traces of acid being removed by successive additions of water and evaporation to dryness.

Chromatography and radioautography. The following solvents were used for the separation of amino acids: phenol + water + ammonia sp.gr. 0·880 (80 + 20 + 0·3, by vol.); *n*-butanol + acetic acid + water (63 + 10 + 27, by vol.); and methylethylketone + *tert*-butanol + water + diethylamine (40 + 40 + 20 + 4, by vol.; Redfield, 1953).

Protein + cell-wall hydrolysates were separated two-dimensionally in butanol + acetic acid + water followed by phenol + ammonia. Proline and glutamic acid in the pool experiments were separated in methylethylketone + *tert.*-butanol + water + diethylamine followed by butanol + acetic acid + water. The material was applied at the origin in 25 μl. distilled water, containing 50–100 μg. of each amino acid. Whatman No. 3 paper was used throughout.

Radioautographs were prepared as described by Roberts *et al.* (1957). The material applied at the origin was monitored and the X-ray film developed after 10^6 disintegrations/spot had occurred.

Fractionation of culture media. The organisms were separated from the media by filtration through a 2·7 cm. membrane filter, and the filtrate collected in 1/10th volume of 20 % (w/v) TCA solution. The proline content of the media was determined by the method of Troll & Lindsley (1955), and their radioactivity was estimated by plating 25 μl. samples directly on to 1 cm.^{2} planchets and counting as described below. The ^{14}C-glutamic acid in the growth medium was estimated after separation of the amino acid from other radioactive components by ionophoresis. When ^{12}C-glutamate was present in the culture, 50 or 100 μl. portions of medium were streaked in the centre of a strip of ionophoresis paper with appropriate markers and the separation carried out for 3 hr. at 14 V/cm. in pyridine + acetic acid + water buffer (pH 6·0; Ryle, Sanger, Smith & Kitai, 1955). After thoroughly drying the paper, the ^{14}C-glutamate was located by spraying the marker regions with ninhydrin and then eluted into 3·0 ml. distilled water. The eluate was taken to dryness to remove traces of pyridine and redissolved in water. The specific activity of the ^{14}C-glutamic acid was determined from radioactivity measurements and glutamate estimations with ninhydrin. When ^{12}C-glutamate was not present in the medium during growth, 500 μg. ^{12}C-glutamate/ml. was added to the filtrates as carrier.

Estimation of $^{14}CO_2$. A 10 ml. culture was grown in a closed system and bubbled with air at a rate of about 100 ml./min. The effluent gas was passed for a fixed time (usually 5 min.) through 50 ml. N-NaOH containing 0·2 ml. 0·25 M-$KHCO_3$ as carrier. The total carbonate present in the NaOH was precipitated with excess $Ba(OH)_2$, collected on a filter paper disk and the radioactivity determined at infinite thickness. The quantity of carrier used ensured that the radioactive carbon dioxide never constituted more than 5 % of the total amount of carbonate precipitated.

Measurements of bacterial growth. Growth rates were measured turbidimetrically in a Zeiss Spectrophotometer (Model PMQ II) at 450 mμ with a 1·0 cm. light path. Under these conditions, for a culture growing in medium 63 + proline, an optical density of 0·100 corresponds to a dry weight of organism of about 18 μg./ml., and a viable count of about 7×10^7 bacteria/ml. (see Schaechter *et al.* 1958).

Radioactivity determinations. Membrane filters were glued directly to 5·7 cm.^{2} planchets for counting; other samples were plated on 1 cm.^{2} planchets and dried at 65°. Counting was carried out with a Geiger–Müller tube and scaler or with a gas-flow counter (Friesecke & Hoepfner GMBH, Erlangen-Bruck, Germany). A minimum of 10^3 counts above background were collected; background never exceeded 25 % of the total count.

RESULTS

Growth of Salmonella typhimurium *in proline and glutamic acid media*

Preliminary experiments showed that *Salmonella typhimurium* would grow in medium 63 containing proline or L-glutamic acid as sole carbon source at rates of 0·70 and 0·90 doublings/hr., respectively. With proline + L-glutamic acid in the medium 1·1 doublings/hr. were obtained. Organisms growing in proline at the final rate for that amino acid upon addition of L-glutamic acid increased their growth

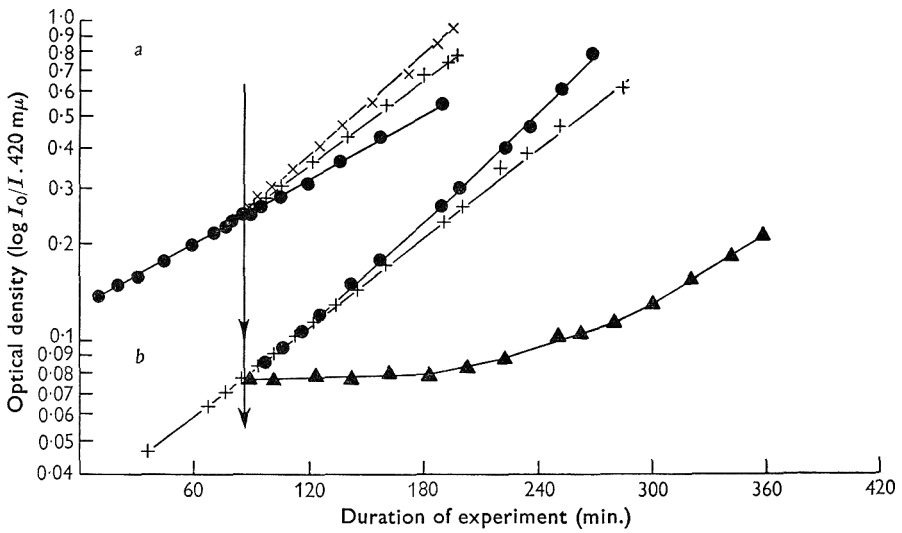

Fig. 1. Growth of *Salmonella typhimurium* in medium 63 containing different carbon sources. (*a*) A culture growing in medium 63 + 500 μg. proline/ml. (●) was filtered at the point indicated by the arrow, washed on the filter with medium 63 and the organisms resuspended in medium 63 containing the following additions: 500 μg. proline/ml. (●), 500 μg. glutamate/ml. (+), 500 μg. proline/ml. + 500 μg. Na glutamate/ml. (×). (*b*) A culture growing in medium 63 + 500 μg. Na glutamate/ml. (+) was filtered at the point indicated by the arrow, washed on the filter with medium 63 and the organisms resuspended in medium 63 with the following additions: 500 μg. proline/ml. (▲), 500 μg. Na glutamate/ml. (+), and 500 μg. proline/ml. + 500 μg. Na glutamate/ml. (●).

rate to the high value characteristic of a glutamic acid + proline mixture. When organisms growing in proline medium were collected by filtration, washed and resuspended in glutamic acid medium, growth was resumed at the rate characteristic of glutamate alone. In both cases the new and higher growth rate was established within about one minute after glutamic acid was introduced (see upper set of curves in Fig. 1). On the other hand, organisms growing in medium containing L-glutamate alone increased their growth rate slowly when proline was added, and replacement of L-glutamate by proline led to a pronounced diauxie. As illustrated by lower set

of curves of Fig. 1, it took several hours before the rate of growth characteristic of proline was established in a medium containing this amino acid as the sole carbon source. Clearly, our strain of *S. typhimurium* can switch readily from growth on proline to growth on glutamate, but not vice versa.

Effect of external concentration of proline or glutamic acid on the growth rate

In several cases the growth rate of bacteria has been shown to be independent of the external concentration of nutrilites over a wide range (e.g. Monod, 1942; Novick, 1955; Novick & Szilard, 1950). This important observation was confirmed

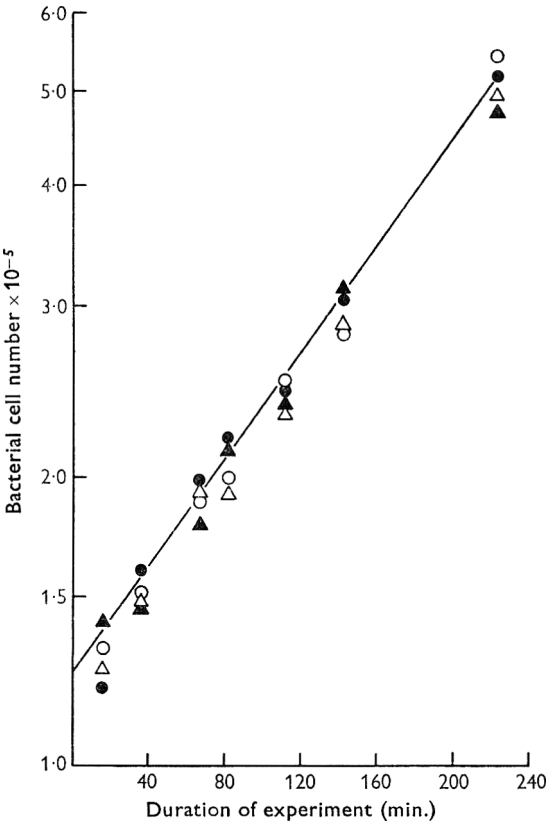

Fig. 2. Growth of *Salmonella typhimurium* in medium 63+different concentrations of proline. Inoculum: about $1{\cdot}2 \times 10^5$ bacteria/ml. Proline concentrations/ml.: 1·0 μg. (●), 10·0 μg. (○), 100 μg. (▲), 1000 μg. (△). Samples measured by viable count.

in our experiments by measuring the growth rate of *Salmonella typhimurium* in media containing proline in concentrations from 1 to 1000 μg./ml. Organisms growing exponentially in medium with 100 μg. proline/ml. were inoculated to a concentration of 10^5 organisms/ml. into media containing 1, 10, 100, and 1000 μg. proline/ml., respectively. By viable counts it was shown that the growth rate of all four cultures was the same over a period of at least two generations (Fig. 2). The same result was obtained when growth was followed turbidimetrically, with inocula of 3×10^7 organisms/ml. in the presence of 20, 60, 200, 600 and 2000 μg. proline/ml.

Similar results were obtained with L-glutamic acid as sole carbon source. Thus within wide limits of concentration, the rate of balanced growth of these organisms was defined by the qualitative composition of the medium.

Investigation of the rate-limiting reaction

Flow of proline to the free amino acid 'pool'. The rate of growth in proline medium might be limited by the rate of flow of proline into the bacterial cells. Experiments were therefore carried out to decide whether the rate of inflow of proline could be

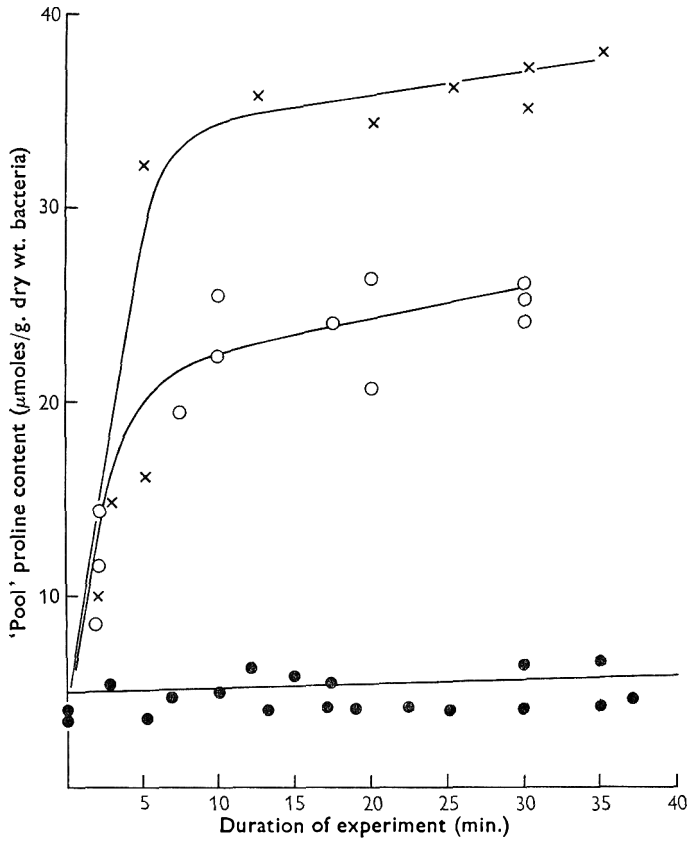

Fig. 3. Effect of changing the external proline concentration on the proline content of the free amino acid pool of *Salmonella typhimurium*.

Organisms were prepared for the experiment by the 'cycling procedure' (see Methods) in medium 63 + 10 μg. proline/ml. The organisms were then separated by filtration and resuspended (at time zero) to an optical density of about 0·05 in medium 63 containing the following concentrations of proline (μg./ml.): 10 (●), 500 (○), 1000 (×). Samples taken at intervals were treated to liberate the free amino-acid pool. The proline content of the pool was measured by the method of Troll & Lindsley (1955).

increased above that required for growth at the characteristic rate. Organisms were grown under approximately steady state conditions in medium containing 10 μg. proline/ml. After 5 cycles of dilution (see Methods), half the culture was poured on to sufficient solid proline to raise the external concentration to 1 mg./ml. The other half of the culture was maintained in medium containing 10 μg. proline/

ml. Samples were taken in rapid succession from both cultures and the pool concentration of proline estimated chemically. After all the samples had been removed, incubation was continued for at least one generation, to ascertain that the growth rate was the same at both proline concentrations. Figure 3 shows the results from a number of experiments of this kind. In all cases, transfer of the organisms to a higher external proline concentration resulted in a rapid increase in the proline pool with no change in the growth rate. The increase was seven- to eight-fold after a shift from 10 to 1000 μg./ml., and about five-fold after a shift from 10 to 500 μg./ml.

These results indicate that, during the short period when the proline pool increased from 15–20 to about 140 μmole/g. the rate of flow of proline into the pool was about 20 % higher than it was during balanced growth. Thus, if we disregard the unlikely possibility that growth was slowed down correspondingly during the build-up of the proline pool, we may conclude that the flow of proline from the medium to the free amino acid pool of the organisms did not restrict the growth rate. Results published by Britten (Britten & McClure, 1961) indicate that a similar deduction can be made for *Escherichia coli*.

Dissimilation of carbon from proline. The degradative pathway for proline is not fully known, and glutamic acid is the only clearly identified product (Stetten, 1955; Vogel, 1955). It has been reported that radioactive proline can be converted to ornithine, citrulline and arginine by *Neurospora crassa*, *Torolopsis utilis* (Srb, Fincham & Bonner, 1950; Fincham, 1953) and *Escherichia coli* (Vogel, Abelson & Bolton, 1953) without the carbon passing through glutamic acid. Our experiments with *Salmonella typhimurium* show that most of the proline-carbon reached the protein of the organisms via glutamic acid. This was studied by measuring the effect of exogenous non-radioactive glutamic acid on the flow of radioactive carbon from ^{14}C-proline to individual amino acids of the cell proteins. The reasoning is as follows. If all the proline-carbon flows through glutamate, the addition of ^{12}C-glutamate should depress to an equal extent the flow of radioactivity to all amino acids in the protein. On the other hand, if certain amino acids were synthesized from proline without involving glutamic acid, these amino acids should be less affected by the addition of non-radioactive glutamate. Thus, if ornithine were synthesized directly from proline, the arginine isolated from the protein should have the same high specific activity as the administered proline. Figure 4 shows the effect of adding 1 mg. ^{12}C-glutamate/ml. to a culture growing exponentially in medium containing 250 μg. ^{14}C-proline/ml. (0·03 μC/μmole). A second part of the culture, to which no glutamate was added, served as a control. Upon addition of ^{12}C-L-glutamate the differential rate of incorporation of radioactive carbon into TCA-precipitable material was immediately decreased to about 35 % of the control value. This diminished rate remained constant for at least three generations.

Samples from both cultures were treated to yield the protein + cell-wall fraction and this was hydrolysed to liberate the free amino acids. Table 1 shows that the flow of radioactivity to various amino acids in the protein, except to proline, was decreased approximately to the same extent by the addition of non-radioactive glutamate. Table 2 shows that the specific activities of certain amino acids including arginine and lysine, are nearly identical. Thus a direct, or preferential, conversion of proline to arginine is unlikely. From the errors involved in an experiment of this

kind, we estimate that as much as 15 % of the carbon from proline could flow by some route other than via glutamate without this being detected.

Given these results, it was of interest to see whether an increase in the external proline concentration led to an increase not only in the proline but also in the glutamic acid content of the free amino-acid pool. The experiment described in the previous section was therefore repeated, the pool material this time being desalted on a Dowex 1 column and the proline and glutamic acid determined chromatographically. As before, an increase in the external concentration of proline from

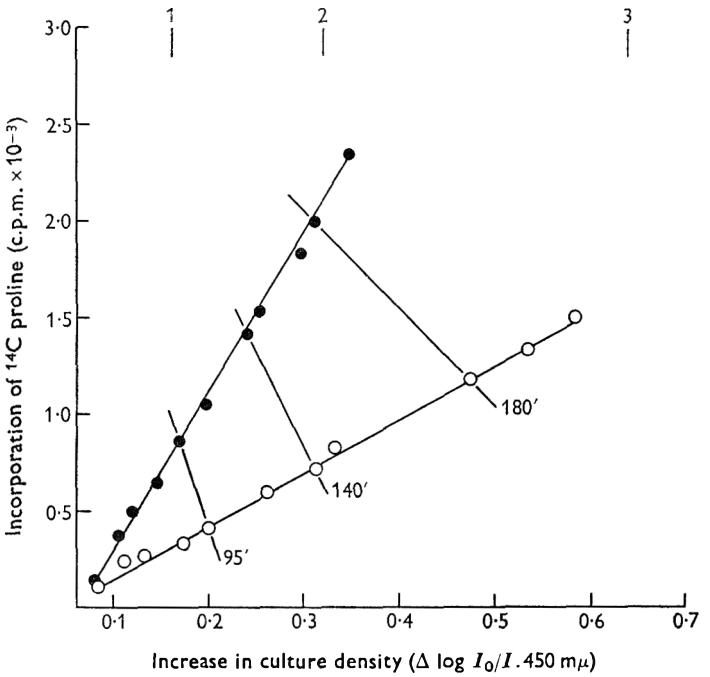

Fig. 4. The effect of addition of ^{12}C-glutamate (1 mg./ml.) on the incorporation of ^{14}C-proline into *Salmonella typhimurium*.

S. typhimurium was grown in medium 63 containing 500 μg. ^{14}C-proline/ml. (0·03 C/mole) alone (○), or proline + 1 mg. glutamate/ml. (●). Samples were taken at intervals into 5 % (w/v) TCA solution at 2° and the radioactive content of the organisms determined by the membrane filter technique (see Methods). Samples for hydrolysis were removed 95, 140 and 180 min., respectively, after addition of ^{14}C-proline. At the top of the frame the figures 1, 2 and 3 indicate generations of growth (doublings) from time of addition of the radioactivity.

10 μg. to 1000 μg./ml. caused an eight-fold increase in the proline content of the pool. In contrast, the glutamic acid pool only increased about 1·3-fold (see Fig. 5). This relative stability of the glutamate pool, together with the fact that changes in the external proline concentration had no effect on the growth rate, indicate that the rate at which proline was converted to glutamic acid was itself independent of the external proline concentration. We have seen that addition of glutamate to the proline medium immediately increased the growth rate, and thus the flux of carbon derived from glutamate. Experiments similar to those presented in Figs. 3 and 5 showed that in this case the glutamic acid pool increased about ten-fold.

Before concluding that the rate of conversion of proline to glutamic acid is independent of the external proline concentration one must make sure that, at high proline concentrations, excess glutamic acid, or products thereof, are not excreted into the medium. This possibility was tested by inoculating exponentially growing organisms into a medium containing 265 μg. ^{14}C-proline/ml., and allowing growth to continue until the proline was exhausted. In this way the metabolism of the organisms could be studied under conditions in which the external proline concentration decreased from 250 μg./ml. to nil. During this period the following factors were measured: (1) the incorporation of radioactivity into whole bacteria; (2) the incorporation of radioactivity into TCA-insoluble cell material; (3) the

Table 1. *Effect of addition of* ^{12}C-*glutamate on the flow of radioactivity from* ^{14}C-*proline to the amino acids of bacterial protein in* Salmonella typhimurium

^{12}C-glutamate (1 mg./ml.) was added to half of a culture of *S. typhimurium* growing in medium 63 containing 250 μg. ^{14}C-proline (0·03 C/mole). The other half was incubated without addition of glutamate. Samples taken as indicated below were treated to yield the protein + cell-wall fractions of the organisms which were hydrolysed to give free amino acids. These were separated by chromatography and their positions located by radioautography. The quantity of radioactivity in each spot was estimated after elution. The results are quoted in relation to a value for proline of 1·0 (see also Fig. 4).

	Medium 63 + ^{14}C-proline				Medium 63 + ^{14}C-proline + ^{12}C-glutamate				
Sample...	1	2	3	Average	1	2	3	Average	Average % of control
Time of sample (min.) ...	95	140	180		95	140	180		
Optical density ...	0·091	0·157	0·228		0·108	0·221	0·373		
Leu/Ileu	1·3	1·2	1·1	1·2	0·45	0·37	0·35	0·39	35
Phe/Val.	1·4	1·4	1·2	1·3	0·45	0·50	0·53	0·47	35
Tyr	0·30	0·35	0·30	0·32	—	(0·13)	0·18	0·15	—
Ala	1·0	0·95	0·87	0·94	0·30	0·28	0·35	0·27	35
Thr	0·25	0·3	0·27	0·27	(0·1)	0·12	0·08	0·11	(24)
Ser/Gly	0·95	0·9	1·15	0·95	0·32	0·36	0·35	0·34	28
Glu	2·1	1·9	1·8	1·95	0·65	0·75	0·80	0·70	37
Lys/Arg	1·7	1·8	1·8	1·78	0·60	0·70	0·77	0·69	39
Asp	1·2	1·4	1·7	1·42	0·40	0·37	0·45	0·41	29
Pro	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	—

Bracketed values: radioactivity too low for accurate estimation. —, sample lost.

Table 2. *Specific radioactivity of certain amino acids isolated from the protein + cell-wall fractions of* Salmonella typhimurium *growing in* ^{14}C-*proline* (250 μg./ml.: 0·03 C/mole) + ^{12}C-*glutamate* (1 mg./ml.)

	Medium 63 + ^{14}C-proline			Medium 63 + ^{14}C-proline + ^{12}C-glutamate		
Sample	1	2	3	1	2	3
	c.p.m. × 10^{-3}/μmol					
Pro	0·30	0·35	0·45	0·34	0·40	0·47
(Pro+	0·30	0·37	0·42	0·34	0·43	0·45)
Glut	0·40	0·44	0·48	0·17	0·18	0·20
Asp	0·36	0·39	0·52	0·17	0·17	0·18
Lys	0·30	0·40	0·43	0·15	0·15	0·19
Arg	0·35	0·45	0·50	0·18	0·17	0·22

Pro+ = Theoretical values for the specific activity of proline calculated from the initial specific activity of the ^{14}C-proline added and the unlabelled inoculum added at the beginning of the experiment. (See also Table 1.)

disappearance of proline from the growth medium; (4) the radioactivity remaining in the medium after treatment with acid to liberate $^{14}CO_2$; (5) the appearance of ^{14}C-glutamate in the medium. Figure 6 shows the results of these measurements. In all cases the differential plots are approximately linear, indicating that the parameters measured were independent of the external proline concentration. No ^{14}C-glutamate was detected in the growth medium.

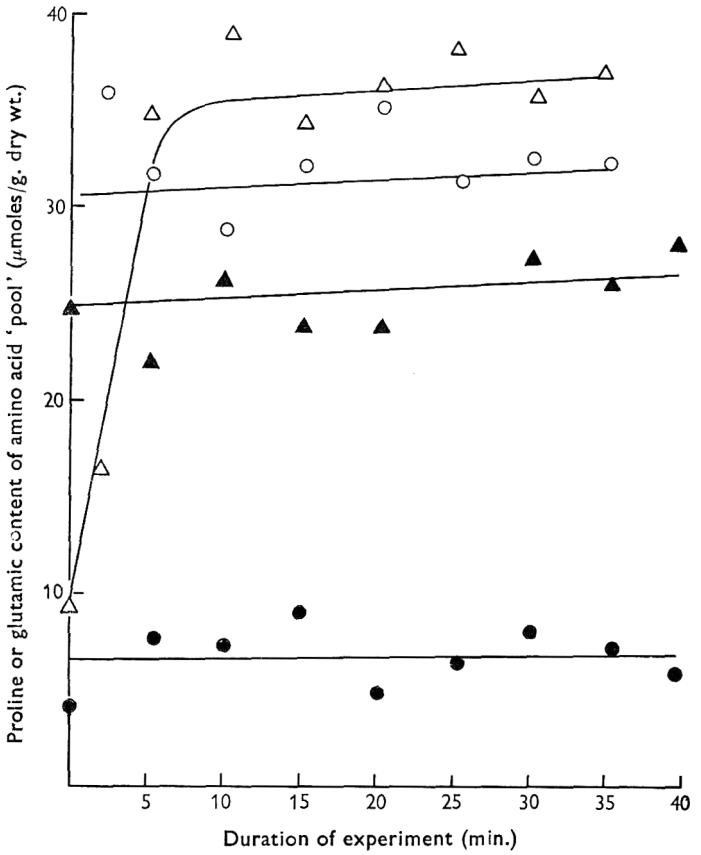

Fig. 5. Effect of changing the external proline concentration on the proline and glutamic acid content of the free amino acid pool in *Salmonella typhimurium*.

The organisms were prepared for the experiment by the 'cycling procedure' (see Methods) in medium 63 + 10 μg. proline/ml., separated by filtration and resuspended to an optical density of about 0·05 in media containing 10 μg./ml. (● and ▲) and 1000 μg./ml. (○ and △) of proline. The proline (△) and (●), and the glutamic acid (▲) and (○) content of the pool was measured after desalting and separation by chromatography.

From the data presented in Fig. 6 estimates of the non-proline radioactivity remaining in the medium at various times were obtained by subtracting from (4) the activity corresponding to the proline still present in the medium. As shown by the two descending lines in Fig. 6 this non-proline activity built up in the medium at a constant differential rate, and at the end of the experiment it amounted to about 20 % of the input activity. The $^{14}CO_2$ produced under these conditions was determined in a special experiment, since the errors involved in calculating the CO_2 production from the data of Fig. 6 are very large. Figure 7 shows that the differential

rate of CO_2 production remained constant until more than 90 % of the proline had been consumed. At this point about 12 % of the input activity had been released as $^{14}CO_2$. Thus, of the generally labelled proline consumed in these experiments about $\frac{2}{3}$ of the carbon were incorporated into cell material and $\frac{1}{3}$ was released into the medium.

In summary, large changes in the external proline concentration have correspondingly large effects on the proline pool in the cells, but no effect on the growth rate of the organisms, and little, if any, effect on the size of the glutamic acid pool or on the pattern of assimilation of carbon from proline. We interpret this to mean

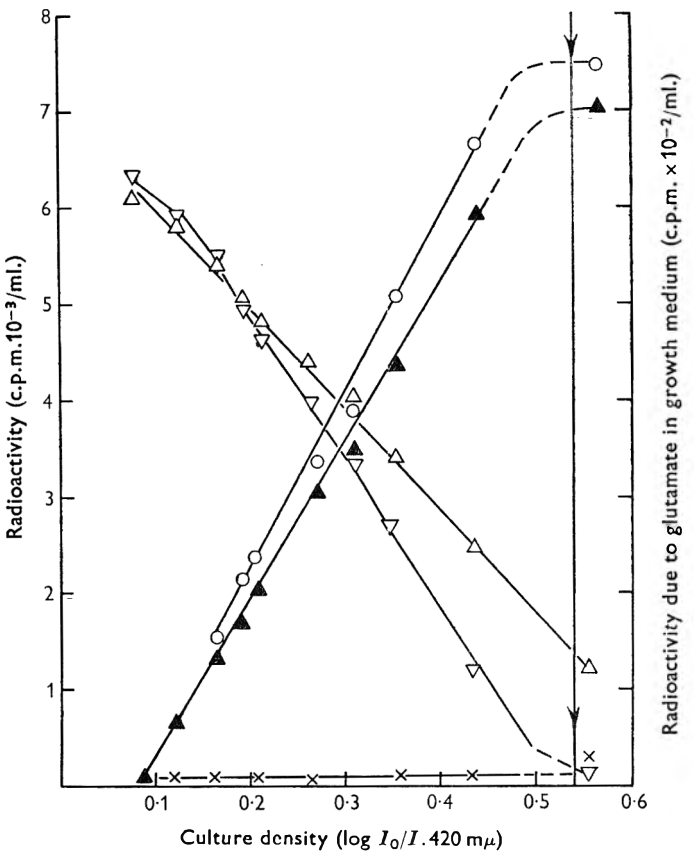

Fig. 6. Analysis of various culture components during the utilization and exhaustion of proline.

Salmonella typhimurium was grown in medium 63 containing 265 μg. ^{14}C-proline/ml. (0·03 C/mole) until the proline was exhausted. Samples of the culture (2·5 ml.) were filtered into 1/10th vol. 20 % (w/v) TCA solution and the concentration of proline and the radioactivity present in the medium estimated directly on 0·5 and 0·05 ml. samples, respectively. The quantity of glutamic acid was estimated on a further 1·0 ml. of the filtrate as described under Methods. The radioactivity in the medium (△) and the radioactivity due to glutamic acid in the medium (×) are plotted directly. The external proline concentration is plotted (▽) after conversion to c.p.m./ml. from the initial specific radioactivity of the proline added to the medium. Further 1·0 ml. samples were filtered to measure the total radioactivity incorporated into the organisms (○) and the radioactivity incorporated into the TCA precipitable portion of the bacteria (▲). The theoretical point of exhaustion of proline is shown by the arrow. Initial suspension optical density 0·08.

that the rate at which proline is converted to glutamic acid is largely responsible for the fact that *Salmonella typhimurium* grows slower with proline than with glutamic acid as the sole carbon source.

This conclusion is based on the following reasoning. At different external proline concentrations a series of steady states of growth are observed which are identical with respect to growth rate, pattern of dissimilation of proline and intracellular concentration of glutamic acid, and which differ only with respect to the intracellular concentration of proline. In these cases the rate of flow of carbon from

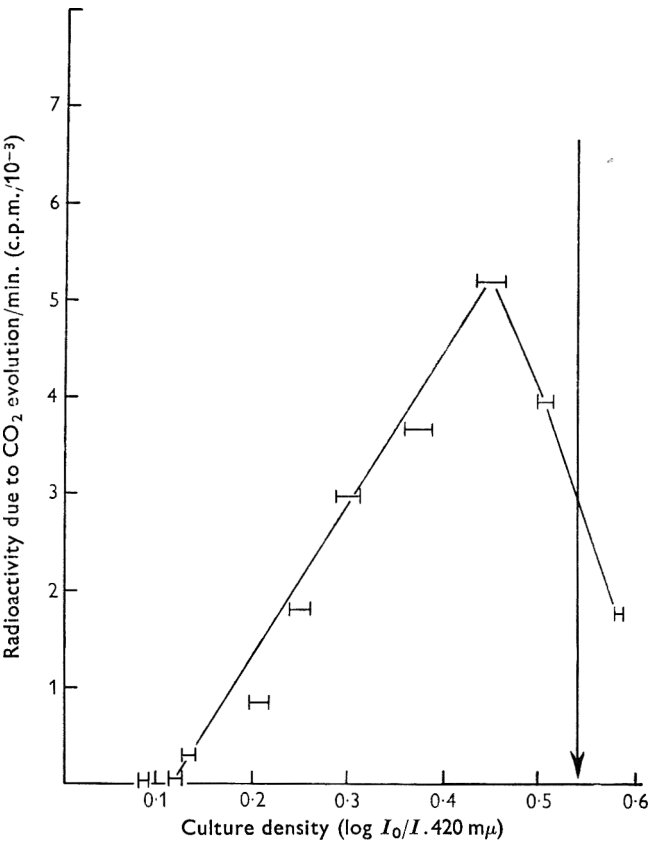

Fig. 7. Liberation of $^{14}CO_2$ during utilization and exhaustion of proline.
Salmonella typhimurium was grown in medium 63 + ^{14}C-proline (250 μg./ml.) (0·03 C/mole) until the proline was exhausted. The culture (initial optical density, 0·115) was kept in a closed system and the total amount of $^{14}CO_2$ liberated in 5 min. periods estimated. The theoretical point of exhaustion of proline is shown by the arrow. The bars across each point represent the increase in culture optical density during the 5 min. period in which the sample was collected.

proline into general cell mass must therefore be the same, and the enzymes responsible for converting proline to glutamic acid must be saturated; i.e. the growth rate must be limited by the capacity of this enzyme system.

It is perhaps surprising that a constant glutamate pool of about 30 μmole/g. should be maintained under these conditions. We feel that the constancy of this pool reflects the fact that the enzymes responsible for converting proline to gluta-

mate seem to be saturated even at low external proline concentrations. In the steady state of growth the intracellular concentration of glutamic acid obviously must assume the value required to maintain the constant flow of glutamate. The observed glutamate concentration (pool size) may therefore be looked upon as a general characteristic of the enzyme system which makes glutamate-carbon available for growth. This interpretation seems reasonable since the addition of glutamate to the medium has the two-fold effect of increasing the growth rate and the size of the glutamate pool. If we assume that this increase in pool size is necessary to bring about an increase in growth rate, it follows that the enzymes responsible for converting glutamate to cell mass are not saturated during growth with proline as sole carbon source.

Effect of the synthesis of the proline-degrading enzymes on growth rate

As mentioned above, the proline-degrading enzymes might constitute so large a fraction of the cell protein that the relative size of other enzyme systems, and thereby the growth rate, was decreased. A restriction of this kind might operate in addition to the limitations discussed above. If the synthesis of the proline-degrading enzymes is to account wholly, or in part, for the fact that the growth rate on proline is less than that on glutamate, the synthesis of these enzymes should cease or be markedly decreased on addition of glutamate to a proline culture. Unfortunately, no reliable method is available which directly measures the quantity of proline-degrading enzymes present in these organisms. An indirect estimate can, however, be obtained by measuring the effect of addition of ^{12}C-glutamate on the incorporation and dissimilation of ^{14}C-proline. As we have seen (p. 276 and Fig. 4), addition of glutamate to a culture growing with ^{14}C-proline led to an immediate increase in the growth rate and to an immediate decrease in the differential rate of incorporation of radioactivity into TCA-insoluble cell material. Since this decreased rate of uptake remained constant over more than three generations, the addition of glutamate did not completely inhibit the function of or repress the production of the proline-degrading enzymes.

A more detailed study of the effect of added glutamic acid on the amount of radioactivity passing through the proline-degrading enzymes was made as follows. A culture was grown in medium containing 100 μg. ^{14}C-proline (0·03 C/mole) and the dissimilation of radioactivity followed in the presence and the absence of 1 mg./ml. of ^{12}C-glutamate. As in the experiment of Fig. 6, the following parameters were measured: (1) and (2) the incorporation of radioactivity into whole organisms and into TCA-insoluble cell material, respectively; (3) the disappearance of proline from the medium; (4) the disappearance of radioactivity from the medium; (5) the appearance of ^{14}C-glutamate in the medium. These measurements permitted a calculation of the differential rate of appearance of radioactivity due to compounds other than proline. In the presence of glutamate the rate of incorporation of ^{14}C-proline was about 30 % of that achieved in the control culture. Similarly, glutamate depressed the quantities of radioactivity appearing as compounds other than proline. Under these conditions, however, the glutamate in the growth medium became radioactive and the flow of radioactivity by this route amounted to about 12 % of the incorporation of radioactivity into TCA-insoluble cell material. Figure 8 summarizes the results of this experiment.

Our experiments show that the addition of glutamate to cultures grown with proline alone resulted in an immediate partial inhibition of the function of the proline-degrading enzymes. Thus the rate of production of glutamic acid from radioactive proline, taking into account the activity appearing in the medium, was immediately decreased to a fixed value of about $\frac{1}{3}$ when ^{12}C-glutamate was added. The constancy of this value shows that the synthesis of the proline-degrading

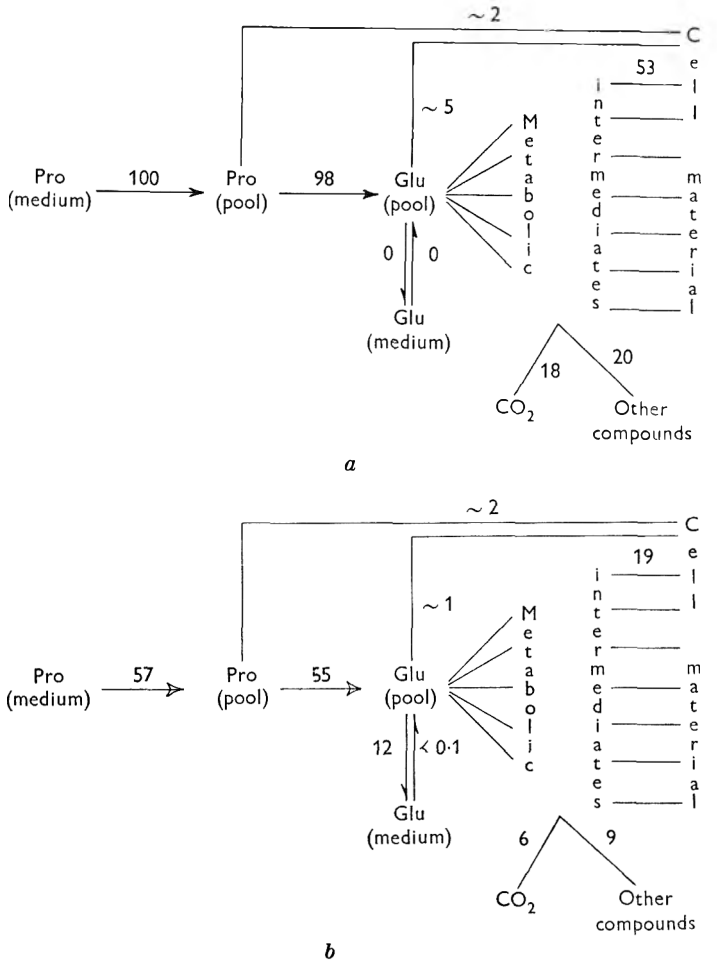

Fig. 8. Diagram to show the flow of carbon from ^{14}C-proline through cultures of *Salmonella typhimurium* growing in medium 63 + ^{14}C-proline (500 μg./ml.; 0·03 μC/μmole) (Fig. 8*a*) and medium 63 + ^{14}C-proline + ^{12}C-glutamate (500 μg./ml.). The differential rate of uptake of radioactivity by organisms growing in proline as sole carbon source is taken as 100. When organisms grow on mixtures of proline and glutamate, part of the carbon required for growth comes from the glutamate, and the differential rate of uptake of radioactivity for the proline in the medium is decreased.

enzymes was not completely repressed. Partial repression cannot be excluded, but the fact that the differential rates of disappearance and of dissimilation of ^{14}C-proline remained constant for several generations after addition of glutamate argues against this possibility.

Considering that the growth rate is immediately increased when glutamate is added to a proline culture, and since a case cannot be made for simultaneous repression of the synthesis of a sizable fraction of the enzyme protein of the cell, our conclusion is that the rate of conversion of proline to glutamic acid is the factor mainly responsible for the cells growing more slowly with proline than with glutamic acid as sole carbon source.

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The Rate of Growth of *Salmonella typhimurium* with Individual Carbon Sources Related to Glucose Metabolism or to the Krebs Cycle

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SUMMARY

Salmonella typhimurium was grown in a number of media containing compounds related either to the tricarboxylic acid (Krebs) cycle or to glucose metabolism. Organisms grown on compounds related to the Krebs cycle are pink when looked at by reflected light, whereas those grown on compounds related to glucose are white. Organisms growing in media containing compounds from one group continue to grow when transferred to media containing another compound of the same group, but not when transferred to media containing a compound from the other group. Growth of organisms in a mixture of one compound from each group leads to the formation of white organisms, i.e. the glucose-like state appears to predominate.

INTRODUCTION

An ealier paper has described the changes occurring in *Salmonella typhimurium* when organisms growing exponentially in medium 63 containing proline were transferred to medium 63 containing glutamate as sole carbon source, or vice versa (Maaløe & Richmond, 1961). The experiments reported in the present paper describe the behaviour of the same strain of *S. typhimurium* in shift experiments involving other compounds as sole carbon sources. All the compounds chosen are closely related metabolically to either glucose or the tricarboxylic acid (Krebs) cycle. Organisms growing on compounds related to the Krebs cycle were pink, whereas those grown in compounds related to glucose were white. Further experiments showed that, in general, organisms grown in media containing a compound from one group would continue to grow on transfer to media containing a compound from the same metabolic group, but not when the new medium contained a compound from the other group as sole carbon source. Some preliminary experiments in which bacteria are grown in media containing a mixture of two compounds from different metabolic groups show that the organisms slowly take on the characteristics of the glucose group if incubation in the mixture is continued. The implication of these findings in relation to the enzymic make-up of the organisms is discussed.

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METHODS

Organisms, media and growth conditions. The strain of *Salmonella typhimurium* used in these experiments together with the composition of media and details of culture conditions are described in the previous paper (Maaløe & Richmond, 1961). *Escherichia coli* (ML 30) was used in certain experiments and was grown in media similar to those used for *S. typhimurium*.

Chemical compounds. The substrates added to medium 63 as sole carbon sources were all obtained from the California Corporation, Los Angeles, 63, California, U.S.A. The ^{14}C-L-proline and the $^{59}FeCl_3$ were obtained from the Radiochemical Centre, Amersham, Buckinghamshire, England. The ^{59}Fe was added to medium 63 to give specific activity in the medium of 3650 c.p.m./μg. atom. The proline was purified as described previously (Maaløe & Richmond, 1961) and used at the specific activities indicated in the text.

Membrane filters. Membrane filters (400 mμ mean pore diameter) were obtained from the Membranfilter Gesellschaft, Göttingen, Germany. The filters were found to contain considerable quantities of glycerol which contaminated the growth media used to resuspend organisms after filtration. The presence of glycerol was found to shorten the lag period observed in certain experiments in which organisms were transferred from one growth medium to another. In no case was the presence of glycerol found completely to abolish a lag following a change in growth medium.

Estimation of colour of organisms. About 2×10^{10} organisms were filtered on to a 2·9 cm. membrane filter. The filter was removed from the filter holder, stuck to a glass microscope slide and dried at room temperature for at least 4 hr. Because of the design of the filter assembly (Roberts *et al.* 1957) the organisms were deposited in a circular area of about 1·4 cm. diameter in the middle of the filter.

To measure the colour of organisms during the transition from white to pink (or vice versa), filters obtained as described above were compared with a standard curve prepared by filtering a constant number of organisms made of varying proportions of pink and white cells. For normal purposes the standard curve consisted of six filters varying over 20 % steps from 100 % pink bacteria to 100 % white. The experimental filters were then matched to the standard curves by uninitiated people and the average of at least three independent settings plotted. Observers usually agreed on the position of a filter opposite the standard curve within the limits ± 15 %.

Extraction and estimation of soluble cytochrome components. About 20 ml. culture medium containing the equivalent of 0·5 mg. dry wt. organisms was precipitated with 80 ml. of absolute acetone at $-8°$ and the preparation kept at that temperature. After at least 1 hr. as much of the supernatant acetone as possible was decanted and the precipitated organisms collected by centrifugation at $-8°$. The pellet was washed once with cold acetone, resuspended in *n*-butanol at room temperature, and the organisms treated at 2° in a Waring blender. After 15 min. the organisms were collected by centrifugation at $-8°$ and the butanol layer discarded. Residual traces of butanol were removed by washing (twice) with cold absolute acetone, and the cytochrome extracted from the organisms by treatment for 30 min. with successive 5 ml. portions of distilled water at room temperature. The extracts were separated from the organisms by centrifugation and combined. The quantity of

cytochrome was measured at 416 mμ in cuvettes of 5 cm. light path after addition of 10^{-4} M-$Na_2S_2O_4$ (Tissières, 1956; Richmond & Kjeldgaard, 1961).

Estimation of ^{59}Fe. Samples were prepared for counting as described previously for ^{14}C (Maaløe & Richmond, 1961). Since ^{59}Fe decays to give β particles (0·46 MeV) and γ-radiation (1·3 MeV), the radioactivity was determined with an end window Geiger–Müller counter in the way described for ^{14}C. About 50 % of the β particles and about 5 % of the γ-rays were measured by the equipment used.

RESULTS

Preliminary experiments showed that *Salmonella typhimurium* grown in medium 63 containing Na glutamate or proline as sole carbon source were pink when looked at in bulk by reflected light. Organisms grown on glucose as sole carbon source were white or pale cream under similar conditions. In view of these findings, organisms were grown exponentially in medium 63 (containing KH_2PO_4, NH_4Cl, $MgSO_4$, $FeCl_3$; see Maaløe & Richmond, 1961) containing different carbon compounds as sole carbon sources and the colour of the organisms in the exponential phase of growth observed by the filter technique. The colours of the organisms grown on the different carbon compounds are recorded in Table 1. Two clear classes of organisms were obtained. The first (glutamate-grown) were characterized by their pink colour; the second (glucose-grown) were white. It is striking that the compounds related to white bacteria are those closely related metabolically to glucose; those producing pink bacteria are related to the tricarboxylic acid (Krebs) cycle.

Table 1. *Colour of* Salmonella typhimurium *after about* 2×10^{10} *organisms had been collected on a* 2·9 cm. *membrane filter*

The organisms were grown in medium 63 containing the different single substrates as sole carbon source

White cells (glucose-like)	Pink cells (glutamate-like)
Glucose, mannose, maltose, fructose, glycerol, dulcitol, arabinose	Glutamate, pyruvate, succinate, fumarate, α-ketoglutarate, malate, histidine, proline, alanine

Since it could be shown that pink bacteria contain a higher amount of cytochrome than white bacteria (Richmond & Kjeldgaard, 1961), the incorporation of ^{59}Fe was followed into the trichloroacetic acid (TCA)-insoluble portion of the organisms grown with glucose or Na glutamate as sole carbon source. The organisms were grown overnight from a small inoculum in medium 63 + 500 μg. glucose/ml. or 500 μg. Na glutamate/ml. and ^{59}Fe. In the morning the organisms were collected on a membrane filter and resuspended to an optical density (Zeiss spectrophotometer; see Maaløe & Richmond, 1961) of about 0·05 in fresh medium of the same composition. Samples taken at intervals into a final concentration of 5 % (w/v) trichloroacetic acid (TCA) solution were filtered and radioactivity determined on the filters. It was found (Fig. 1), that, on a differential basis, the uptake of ^{59}Fe by glutamate-grown organisms was about 1·8 times the rate of uptake by glucose-grown bacteria. As the specific activity of the ^{59}Fe added in the experiment was

known, the iron content of glucose and glutamate grown bacteria could be calculated as 0·02 and 0·036 %, by weight. Assuming an approximate value of 0·5 % for the iron content of cytochromes (see Paul, 1951; Tissières, 1956; Takahashi, Titani & Minakami, 1959; Horio *et al.* 1960), these figures correspond to a total cytochrome content of 4 and 7·2 %, respectively. Extraction of the soluble cytochrome components, however, showed that glutamate from bacteria contained about 0·8 % of their dry weight in this form, whereas no significant quantity of soluble cytochrome

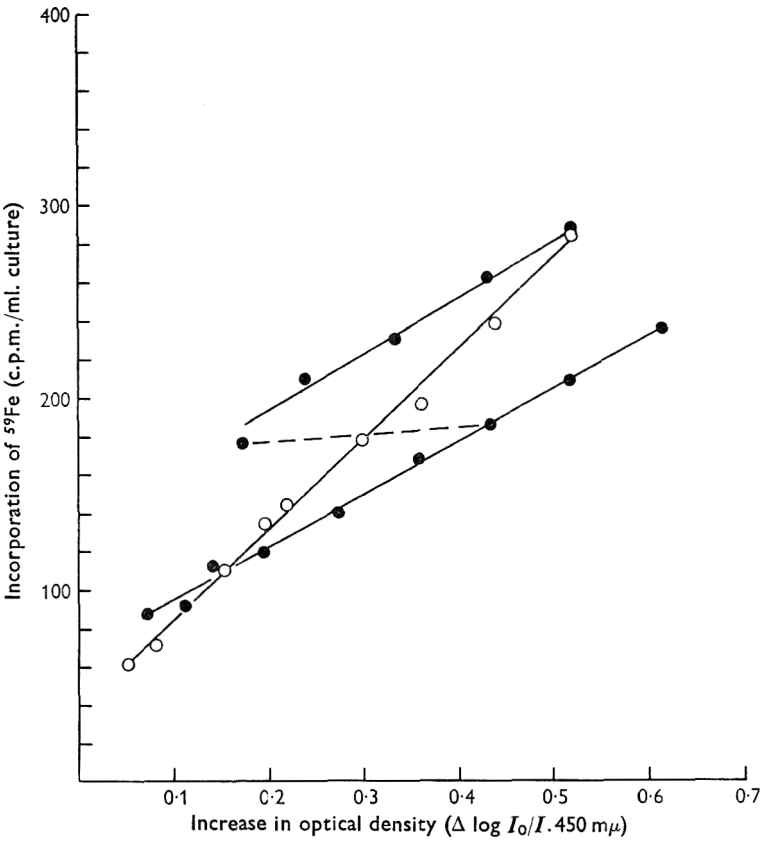

Fig. 1. Incorporation of ^{59}Fe into *Salmonella typhimurium* growing in medium 63 containing ^{59}Fe (3,650 c.p.m./μg. Fe) and either 500 μg. Na glutamate/ml. (○), or 500 μg. glucose/ml. (●). At optical density = 0·435, half the glucose culture was diluted to an optical density = 0·155 with fresh medium 63 + glucose and ^{59}Fe at the same specific radioactivity as the original medium (see dashed line).

was detected in extracts of glucose-grown bacteria. It seems possible, therefore, that the total cytochrome content of glucose-grown bacteria might be much lower than suggested by the radioactive iron incorporation experiments, where some at least of the iron uptake might be due to synthesis of other iron-containing proteins such as catalase.

Transfer of bacteria from one growth medium to another

The use of the membrane filter technique allowed the transfer of organisms growing in the presence of one carbon source to another medium within 2 min. and with a carry over of less than $1/10^5$. This technique was, therefore, used to study the effect of transferring organisms which were growing exponentially on a compound from one class to a medium containing another compound of the same class, or to a compound of the other class. Fig. 2 shows a typical example. Organisms growing exponentially in medium 63 + 500 μg. glutamic acid/ml. were filtered and resuspended

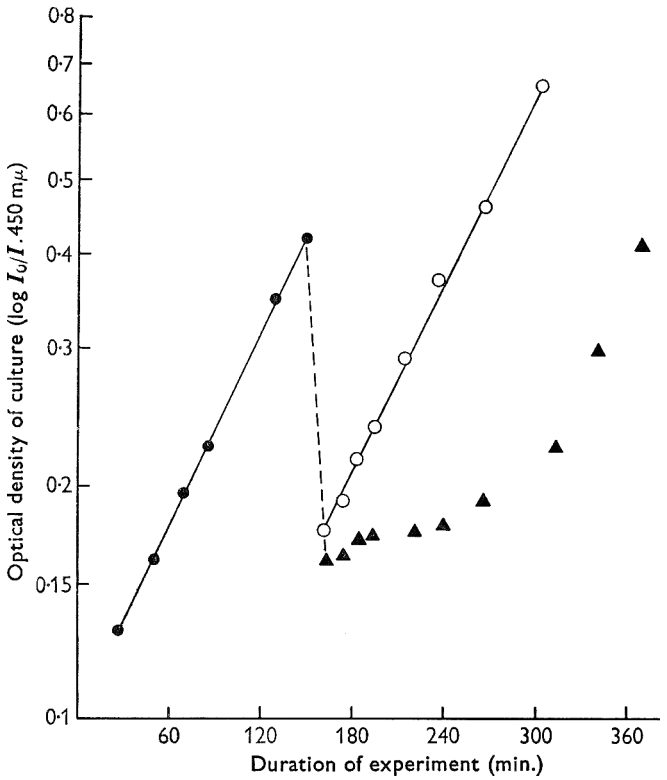

Fig. 2. Growth curves obtained on shifting *Salmonella typhimurium* from medium 63 + Na glutamate to similar medium + glucose. A culture growing exponentially in medium 63 + 500 μg. Na glutamate/ml. (●) was filtered and the total yield of organisms divided between two culture flasks containing medium 63 + 500 μg. Na glutamate/ml. (○), or 500 μg. glucose/ml. (▲).

in medium 63 + 500 μg. succinate/ml. or medium 63 + 500 μg. glucose/ml. Transfer to succinate led to an immediate resumption in growth at the full rate characteristic of succinate. The organisms transferred to glucose did not grow appreciably for about 2 hr., and thereafter the final rate for glucose-grown bacteria was not reached for about 4 generations. Control experiments showed that contamination of the glucose culture with traces of glycerol from the filters did not affect the length of the lag. Table 2 summarizes similar shifts carried out with other combinations of substrates. The basic pattern that is established is that organisms transfer smoothly to substrates of their own class, but not to substrates of the other class. Certain

exceptions are evident (see cases marked by asterisk in Table 2). Bacteria grown on substrates related to the Krebs cycle did not grow immediately when transferred to proline or histidine as sole carbon source, although they are members of the same class. Histidine-grown bacteria did not transfer smoothly to proline, nor proline-grown bacteria to histidine. Since growth on proline or histidine requires the presence of the relevant degrading enzymes which are not present in glutamate-grown bacteria (Maaløe & Richmond, 1961; Richmond, unpublished observations), the lag occurring with these transfers almost certainly represented the time taken

Table 2. *Effect of shifting cultures of* Salmonella typhimurium *from medium* 63 *containing one compound as sole carbon source to another*

	1st carbon source (glucose-like class)			1st carbon source (glutamate-like class)		
	1st carbon source	2nd carbon source	Lag	1st carbon source	2nd carbon source	Lag
2nd carbon source (glucose-like class)	glucose	mannose	–	glutamate	succinate	–
	glycerol	glucose	–	proline	succinate	–
	glucose	glycerol	+*	histidine	α-ketoglutarate	–
	mannose	glucose	–	proline	pyruvate	–
	fructose	glucose	–	glutamate	mannose	+
	arabinose	glucose	–	malate	glucose	+
	glucose	arabinose	+*	α-ketoglutarate	glucose	+
2nd carbon source (glutamate-like class)	glucose	succinate	+	histidine	proline	+*
	glucose	glutamate	+	proline	histidine	+*
	glucose	histidine	+	malate	succinate	–
	glucose	alanine	+	malate	glutamate	–
	glutamate	proline	+	α-ketoglutarate	malate	–
	glutamate	histidine	+	glutamate	proline	+*
	proline	glutamate	–	proline	glutamate	–
	histidine	glutamate	–	histidine	glutamate	–
				histidine	glucose	+
				proline	glucose	+
				glutamate	glucose	+
				succinate	glucose	+

* Cases where a lag occurs after shift experiments involving two carbon sources from the same class. These are probably due to the time required to form specific degrading enzymes (see text).

for the organisms to synthesize sufficient of the substrate-degrading enzymes to support growth. In the glucose class, a similar lag was found on transfer to glycerol or arabinose, but not in the reverse direction. This was probably due to the need to make the arabinose- or glycerol-degrading enzymes.

Effect of addition of glucose to cultures growing in medium 63 + L-*glutamate*

Glucose is known to repress the formation of a number of enzymes (see Magasanik, 1957). It was therefore of interest to see whether addition of glucose to a culture growing exponentially in medium 63 + glutamate would result in any change in the colour of the organisms. A culture was grown in medium 63 + 500 μg. Na glutamate/ml., and after about three generations of exponential growth the culture was divided into two parts and sufficient solid glucose added to one part to bring the final concentration to 500 μg. glucose/ml. Samples (containing about 2×10^{10} bacteria), taken at intervals from both cultures after the addition of glucose, were filtered and

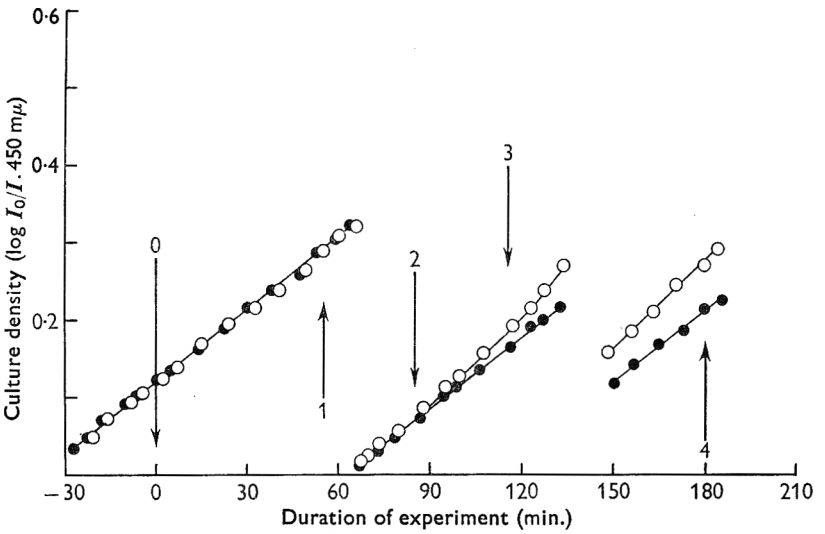

Fig. 3. The effect of addition of glucose on the growth rate (this figure) and colour (Fig. 4) of a culture of *Salmonella typhimurium* growing exponentially in medium containing glutamate. Cultures growing in medium 63 + 500 μg. Na glutamate/ml. (●) and in medium 63 + 500 μg. Na glutamate + 500 μg glucose/ml. (○). Glucose added at $t = 0$. The cultures were diluted with fresh media as appropriate at $t = 67$ and $t = 133$. Samples were taken for colour determinations (see Fig. 4) at $t = 0$, 55, 85, 115 and 180 (see arrows).

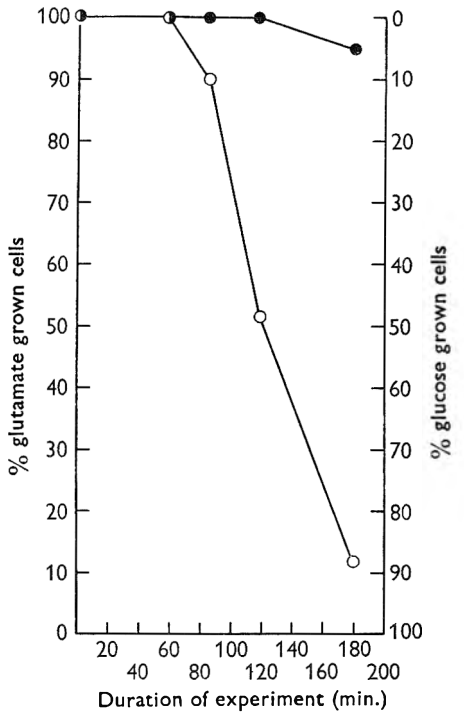

Fig. 4. Samples containing approximately 2×10^{10} organisms were taken at the times indicated in Fig. 3 and filtered as described in the methods section. The points plotted represent the 'colours' of the filters from the average of six different settings achieved by uninitiated people. Organisms grown in glutamate alone (●); organisms grown in glutamate + glucose (○).

the colour of the filtered bacteria compared with a standard curve of filtered bacteria prepared as described in Methods. After the cultures had doubled in optical density from the point of addition of glucose, they were adjusted to the original optical density by the addition of medium 63 containing either 500 μg. Na glutamate/ml. or 500 μg. Na glutamate/ml. + 500 μg. glucose/ml., as appropriate. Sampling was continued, and after a further doubling of the optical density, the cultures were once more diluted to the original optical density. The results of this experiment are shown in Fig. 3. Bacteria growing in Na glutamate continued to grow at the final rate for that medium, and the organisms remained pink throughout the experiment.

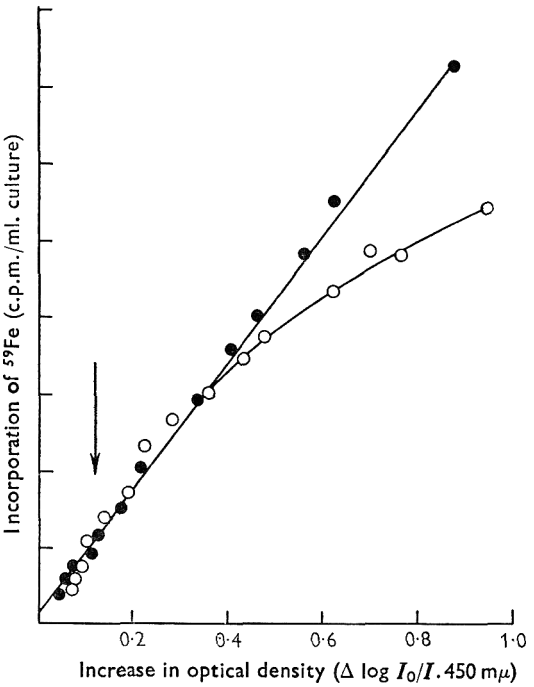

Fig. 5. The effect of addition of glucose on the incorporation of ^{59}Fe into *Salmonella typhimurium* growing in medium 63 + Na glutamate. Glucose added at optical density = 0·12 (see arrow). Culture growing in medium 63 + 500 μg. glutamate/ml. (●) or medium 63 + 500 μg. glutamate/ml. + 500 μg. glucose/ml. (○).

Addition of glucose led to an increase in the growth rate, but this was only detectable about one generation after the glucose was added. Subsequently the growth rate on the glucose + glutamate mixture continued to increase until about 2–3 generations after the addition of the glucose (see Fig. 3). No change of colour from that characteristic of glutamate-grown bacteria was detected for about one generation. Thereafter, the organisms gradually became paler (Fig. 4). When the effect of addition of glucose on the incorporation of ^{59}Fe into cultures growing on medium 63 containing ^{59}Fe and L-glutamate (500 μg./ml.) was followed in a similar way (Fig. 5), it was found that the differential rate of incorporation was not affected for about one generation; thereafter it decreased to a rate characteristic of growth in medium 63 + glucose as sole carbon source (see Fig. 5). It seems, therefore, that addition of glucose to a culture growing in medium 63 + glutamate lead to a shift to the

glucose-grown state even when glutamate remained in the medium; but the onset of the shift could not be detected in the growth curve or the uptake of ^{59}Fe for about one generation. This is probably related to the fact that the organisms grown on glutamate as sole carbon source did not seem to be able to metabolize glucose immediately (see Table 2 and Fig. 2).

The following experiment further substantiates this point as well as showing that glucose had a similar effect in cultures growing in medium 63 + proline. A culture growing exponentially in medium 63 + 500 μg. proline/ml. was filtered and the organisms resuspended in three batches of medium 63 with the following additions

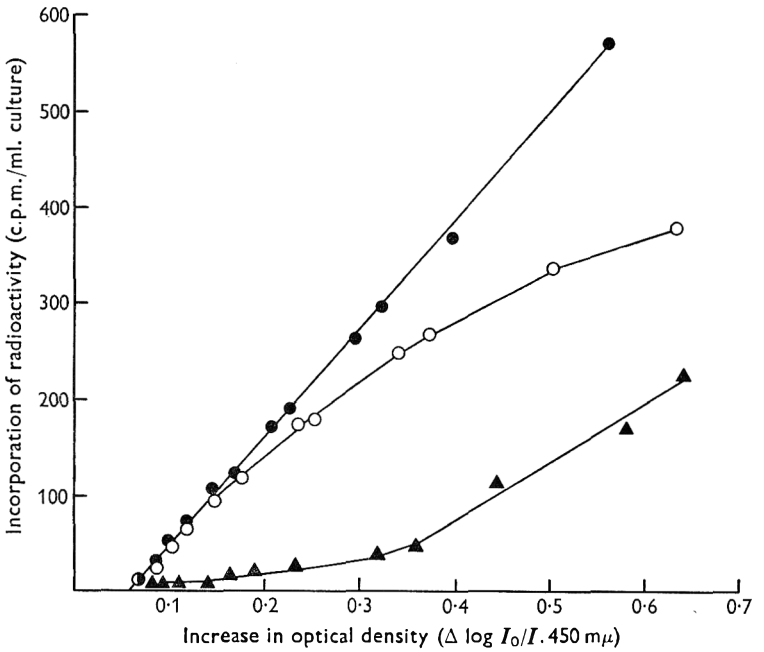

Fig. 6. The effect of addition of glucose to a culture of *Salmonella typhimurium* growing exponentially in medium 63 + proline. A culture growing exponentially in media 63 + proline was filtered and the organisms resuspended in media of the following composition: (1) medium 63 + 500 μg. ^{14}C-proline/ml. (●); (2) medium 63 + 500 μg. ^{14}C proline/ml. + 500 μg. ^{12}C-glucose/ml. (○); (3) medium 63 + 500 μg. ^{12}C-proline/ml. + 500 μg. ^{14}C-glucose/ml. (▲). Specific radioactivity: proline: 11·5 c.p.m./μg.; glucose: 8·8 c.p.m./μg.

(all 500 μg./ml.): (1) ^{14}C proline; (2) ^{14}C proline + ^{12}C glucose; (3) ^{12}C proline + ^{14}C glucose. Samples taken at intervals from each culture were precipitated with an equal volume of cold 10 % (w/v) TCA solution and the incorporation of radioactivity followed by the filter technique (Fig. 6). Culture (1) gave the final differential rate of ^{14}C proline incorporation for proline-grown bacteria. Culture (2) showed that the addition of ^{12}C glucose to cultures growing in proline lead to a decrease in the differential rate of proline incorporation, but that little effect on this rate was detectable for at least one generation after the addition of glucose. Culture (3) confirmed that the differential rate of incorporation of glucose by proline-grown bacteria was very low for about one to two generations after the addition of glucose, but that the rate slowly increased as the utilization of proline decreased. Since glucose had little effect on the flow of radioactivity from ^{14}C proline to the cell for

about one generation, it follows that glucose did not cause immediate complete regression of the formation of the proline-degrading enzymes. However, continued incubation in media containing glucose decreased the ability of the organisms to utilize proline to about 5 % of the original rate after about five generations. This is very similar to the effect of glucose on the behaviour of cultures growing in glutamate (see above), but markedly different from the effect of glutamate on bacteria growing in medium 63 + proline (Maaløe & Richmond, 1961).

Transfer of Escherichia coli *between media containing single compounds as sole carbon sources*

Since lags occurred after the transfer of *Salmonella typhimurium* from media containing substrates related to glucose to substrates related to the Krebs cycle, it was of interest to see whether this was a phenomenon found in another

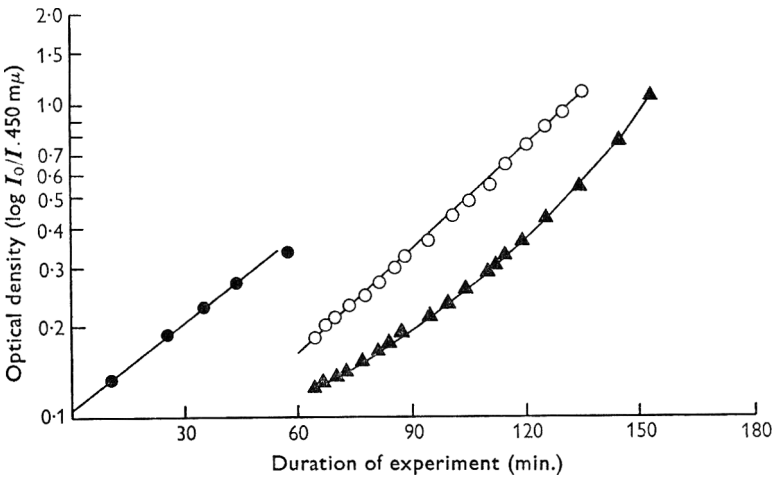

Fig. 7. Growth curves obtained by shifting *Escherichia coli* (ML30) from medium 63 + Na malate (500 μg./ml.) (●) to medium 63 containing Na succinate (500 μg./ml.) (○), or glucose (500 μg./ml.) (▲). Culture filtered and washed at t = 123 min.

organism capable of rapid growth on the simple defined medium 63. To test this, a culture of *Escherichia coli* (ML 30) was grown up overnight in medium 63 + 500 μg. Na malate/ml. In the morning the culture was transferred to fresh medium of the same composition until growth was exponential. After about four generations under these conditions the culture was filtered and divided between two portions of medium 63 containing 500 μg. glucose and 500 μg. Na succinate/ml., respectively.

The subsequent growth of these cultures (Fig. 7) was followed for about 3 hr. The culture transferred to succinate grew immediately at the full rate characteristic of succinate. The culture transferred to glucose grew immediately but with a slowly increasing differential rate. It took at least two generations for the growth rate to reach the full rate characteristic of *Escherichia coli* growing in medium 63 + glucose. Other transfers were carried out, between succinate → glucose, glutamate → glucose and glucose → succinate. In all cases the organisms took up to two generations before they reached the rates characteristic of the second substrates, whereas transfers between succinate → malate, succinate → fumarate and succinate →

alanine led to immediate growth at the full rate in the new medium. It follows that *Escherichia coli* shows the same general response as *Salmonella typhimurium* to these changes in growth medium, but that the effects are much less dramatic.

DISCUSSION

Growth of this strain of *Salmonella typhimurium* with various substrates as sole carbon source led to the formation of organisms of two main types. Bacteria grown on compounds related metabolically to glucose were white and had a relatively low cytochrome and inorganic iron content. On the other hand, bacteria grown with substrates related to the Krebs cycle were pink, had a relatively high cytochrome and iron content, and showed the presence of a soluble cytochrome component (C-type-551: Richmond & Kjeldgaard, 1961) which was either absent, or present in very low concentrations in the glucose-grown bacteria. Fluctuations in the cytochrome content of micro-organisms have been reported by a number of workers (see review by Smith, 1954), but were usually found under conditions in which the oxygen tension in the growth medium was varied (e.g. Moss, 1952; Schaeffer, 1952*a*, *b*; Lenhof & Kaplan, 1953; Chaix & Petit, 1956, 1957; Rosenberger & Kogut, 1958; Heyman-Blanchet & Chaix, 1959). Changes in growth medium have been reported to alter the cytochrome content of cells, but these experiments were usually carried out in complex media in which it was difficult to assess the results (e.g. Keilin, 1933; Gary & Bard, 1952).

It is now generally recognized that the Krebs cycle is one of the main pathways whereby bacteria obtain the energy necessary for growth (see Krebs & Kornberg, 1957). However, it is not the only one, since Roberts *et al.* (1957) showed that when *Escherichia coli* grew on glucose only 30 % of the total amount of CO_2 evolved by the organism came from the Krebs cycle and that, under these conditions, the cycle plays a predominantly synthetic role. As most of the CO_2 evolved came from the C_1 position in glucose, the oxidation of glucose in these organisms probably occurred via the glucose shunt pathway (see Krebs & Kornberg, 1957). Under these circumstances, therefore, it is tempting to think of the two classes of organisms encountered in the present work as characterized fundamentally by the methods in which they derive their energy. Thus organisms of the pink (glutamate-grown) group could derive their energy from the operation of the Krebs cycle and the pink colour is due to a high content of cytochrome, which is known to be associated with some enzymes of the Krebs cycle. On the other hand, the white (glucose-grown) cells could derive their energy from the pathways of glucose degradation: either glycolysis, the glucose shunt pathway or some unidentified pathway. The experiments in which bacteria were transferred from one media to another show that, in general, organisms grown on a compound related to the Krebs cycle did not grow immediately when transferred to media containing a compound from the glucose group as sole carbon source, and vice versa. This was probably because the organisms did not contain the enzymes necessary for growth in the new medium and the lag represented the time taken to synthesize them. Since the system to be synthesized is to be the main energy producing system of the organisms in their new environment, the energy for making these enzymes must, initially, have to come from incidental reactions. This, presumably, accentuates the length of the lag observed, which

may be up to six generations before the full final growth rate in the new medium is attained.

Since addition of glucose to cultures growing in glutamate led to a switch to the 'glucose-like' state and the loss of the ability to utilize glutamate, the glucose-like state must predominate over the glutamate-like. This is a situation analogous to the 'glucose-effect' found during the synthesis of a number of bacterial enzymes (Magasanik, 1957). The finding that, in the present experiments with *Salmonella typhimurium*, the effect was not instantaneous, is presumably due to the fact that glucose was not immediately metabolized when it was added to cultures growing with glutamate or proline as sole carbon source.

The experiments carried out with *Escherichia coli* suggest that a similar situation may occur with these organisms, but the results are not nearly so clear cut. One possible interpretation of these results is that many bacteria can organize their enzymic make-up in a number of ways centred about different methods of deriving energy for growth, and that in some organisms the balance between the various pathways is closer than in others. In the case of the strain of *Escherichia coli* studied here, both the Krebs cycle and the glucose-degrading systems seem to be present in the same cell at the same time and subject only to minor fluctuations. With our strain of *Salmonella typhimurium*, the organism seems to be much more committed to one or the other type of metabolism and the concomitant enzymic make-up.

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One-step Growth Curves for Inclusion Blennorrhoea Virus in HeLa Cell Monolayers

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SUMMARY

When incubated in tissue-culture medium, inclusion blennorrhoea virus lost infectivity for HeLa cells at 37° but not at 30°. The rate of adsorption of virus to HeLa cell monolayers was dependent on temperature and on the volume of the inoculum. When the volume of medium was minimal, the virus was adsorbed before a detectable proportion was inactivated. Adsorption was complete after 7–8 hr. at 30° and 5–6 hr. at 37°.

At 30° the intracellular virus went into eclipse and maturation was retarded. When the temperature was raised to 37° maturation was rapid so that, for practical purposes, replication was synchronized during this period. In singly infected HeLa cells, at 37° infective virus was not detected for 22–23 hr., after which the progeny increased exponentially until at 34–38 hr. 35–60 infectious particles/infected cell were formed. The total number of particles/inclusion seen in Giemsa-stained preparations exceeded the number of infectious units/inclusion.

After 42–48 hr. infective virus was found in the supernatant and the number of intracellular infectious units began to decrease. Unlike vaccinia, another large virus, the virus progeny did not directly infect adjacent cells.

INTRODUCTION

The technique for assaying inclusion blennorrhoea and trachoma viruses in HeLa cell monolayers described by Furness, Graham & Reeve (1960), slightly modified, was used to study the adsorption and intracellular growth of inclusion blennorrhoea virus in HeLa cell monolayers.

METHODS

Virus. The LB1 strain of inclusion blennorrhoea virus (Jones, Collier & Smith, 1959) was grown in HeLa cell monolayers in 250 ml. Pyrex feeding bottles (Furness *et al.* 1960). Suspensions were prepared by resuspending monolayers in 10 ml. medium 68–72 hr. after infection, transferring them to a 50 ml. centrifuge tube cooled in ice water and releasing the virus by treatment for 4 min. with ultrasonic vibrations. For this purpose a stainless-steel probe of an M.S.E.—Mullard ultrasonic disintegrator with a power output of 60 W. at a frequency of 20 kc./sec. was inserted in the cell suspension. The suspensions contained $2{\cdot}5–5 \times 10^7$ infectious units/ml.

HeLa cell monolayers. These were grown in Hank's balanced saline solution

containing 0·5 % (w/v) lactalbumin hydrolysate, 10 % (v/v) human serum, 5 % (v/v) calf serum and 100 μg./ml. streptomycin; the sera were inactivated by heating at 56° for 1 hr. HeLa cell monolayers grown on coverslips in Leighton tubes were used in all quantitative experiments (Furness *et al.* 1960).

Virus assay. The medium covering the HeLa cell monolayers was replaced with 0·25 ml. fresh medium and infected with 0·1 ml. of a suitable dilution of virus. The virus was allowed to adsorb for at least 6–7 hr. at 37°. Thereafter 1·5 ml. medium was added to each monolayer. After incubation for 62–66 hr., the coverslips were removed, stained with Giemsa and the inclusions in thirty fields counted at a magnification of ×240 (Furness *et al.* (1960). Cultures were incubated at 37° unless otherwise stated.

One-step growth curves. Monolayers covered with 0·25 ml. medium were infected with 0·1 ml. of a virus dilution capable of infecting between 300 and 800 of the cells in an area of thirty fields after adsorption for 2 hr. at 30°.

After the virus had been allowed to adsorb to the monolayers for 2 hr. at 30° unadsorbed virus was removed by washing with phosphate buffered saline (Dulbecco & Vogt, 1954) at 30°. To count the number of cells infected, five monolayers were covered with 2 ml. medium and the inclusions counted after incubation for 68–72 hr. at 37°. The remaining monolayers were covered with 1 ml. medium and incubated at 37° to allow maturation of the virus. Released and intracellular inclusion forming units were determined during replication as follows. At intervals, four monolayers were chosen at random and the supernatants pooled and titrated to obtain the number of infectious units released from the cells. The cells themselves were resuspended in a total of 4 ml. medium, disintegrated with ultrasonic vibrations and assayed to determine the number of intracellular infectious units.

RESULTS

The effect of temperature on the viability of inclusion blennorrhoea virus. To assess the rate of inactivation of virus at 37°, 4 ml. of a suitable dilution of virus prepared in medium at pH 6·7–7·1 were incubated in ¼ oz. screw-capped bottles in a waterbath. At intervals 0·1 ml. samples were titrated in HeLa cell monolayers covered with 1 ml. medium. No virus was inactivated in 8–10 hr. at 30°, whereas over 80 % of the virus was killed in 6 hr. at 37°. The virus was not protected by increasing the serum content of the medium from 15 to 45 % (Table 1).

Adsorption of virus to HeLa cell monolayers. To determine the degree of inactivation during the adsorption period, monolayers covered with 1 ml. medium were

Table 1. *The loss of infectivity of inclusion blennorrhoea virus for HeLa cells at* 37°

Suspending medium. Culture medium containing (% serum)	Inoculum (no./ml.)	% infectious units surviving after incubation at 37° for		
		2 hr.	4 hr.	6 hr.
15	$2·8 \times 10^6$	86	28	14
15	$6·7 \times 10^6$	40	33	21
15	$3·0 \times 10^6$	48	21	10
30	$3·0 \times 10^6$	50	24	8
45	$3·0 \times 10^6$	44	22	7

inoculated with 0·1 ml. of a suitable dilution of virus and incubated at 30° or 37° to allow the virus to adsorb to the cells. At intervals, four monolayers were freed from unadsorbed virus by washing with phosphate buffered saline, covered with 2 ml. medium and incubated for 68–72 hr. at 37° to enable adsorbed particles which were still infectious to form inclusions. Compared with the infectious units adsorbed in 8 hr. at 30°, only half the virus formed inclusions at 37° indicating that some infectious units were inactivated. The method of titration of inclusion blennorrhoea virus in HeLa cell monolayers (Furness *et al.* 1960) did not therefore determine the total number of infectious units in the original suspension.

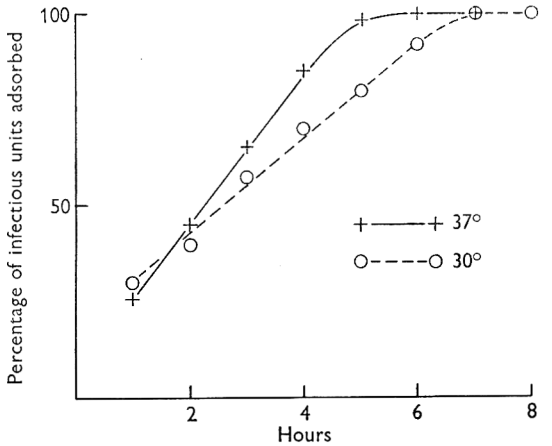

Fig. 1. The rate of adsorption of inclusion blennorrhoea virus by HeLa cells at 30° and 37°.

Since the efficiency of adsorption of polio-virus to cell monolayers is increased by decreasing the volume of the supernatant fluid (McLaren, Holland & Syverton, 1959; Furness, 1961), the experiment was repeated with 0·25 ml. medium; the total volume after inoculation thus being only 0·35 ml. This modification increased the efficiency of adsorption. Similar number of inclusions were formed after adsorption for 7–8 hr. at 30° and 5–6 hr. at 37° (Fig. 1), indicating that the infectious units were adsorbed from this volume before significant inactivation occurred. These results do not appear consistent with the finding that over 80 % of the virus was inactivated in 6 hr. at 37° (Table 1). In the two experiments, the virus was assayed under different conditions which may explain this inconsistency.

To verify that diminution of the volume of supernatant fluid did not affect the accuracy of titrations, the results of four assays were analysed statistically (Furness *et al.* 1960). The random distribution of the inclusions and the accuracy of the titrations were not altered, the standard error being within ± 10 %.

The multiplication of inclusion blennorrhoea virus in HeLa cells. Three one-step growth curves for inclusion blennorrhoea virus are summarized in Fig. 2. In these experiments respectively 1·2, 1·8 and 4·8 % of the cells in the monolayers were infected. The results therefore reflect the behaviour of singly infected cells. After adsorption at 30° for 2 hr., at least 88 % of the adsorbed virus was irrecoverable, the remainder being released as infective virus on disintegrating the cells. Thereafter, the temperature was raised to 37° to allow normal maturation of the virus. Less

than 1 % of the adsorbed virus was infective 4 hr. later. With this virus-cell system, the period during which virtually no virus was recoverable is termed the eclipse stage and is considered to end when each infected cell contains an average of one

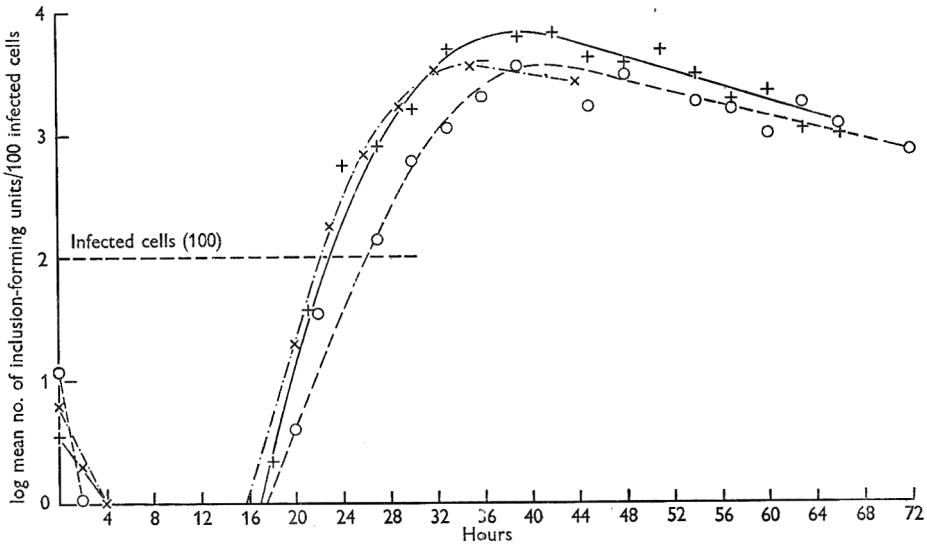

Fig. 2. One-step growth curves of 3 experiments with inclusion blennorrhoea virus in HeLa cell monolayers at 37°.

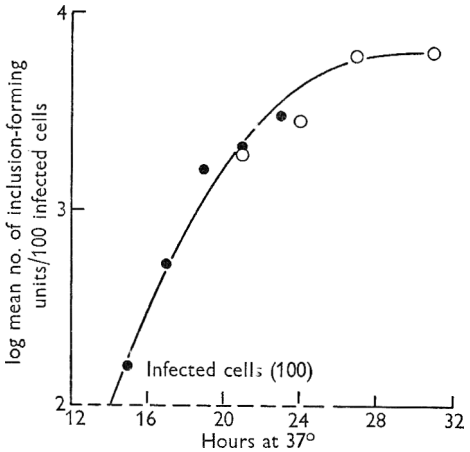

Fig. 3. Effect of pre-incubation for 24 hr. at 30° on one-step growth curve of inclusion blennorrhoea virus in HeLa cell monolayers at 37°. For convenience, this experiment was done in two parts (● and ○).

infectious unit; in these experiments it lasted 22–23 hr. Once replication commenced, the intracellular infectious units increased exponentially until a peak was reached at 34–38 hr. with a yield of 35–60 infectious units/inclusion. From 42–48 hr. until the experiments were ended, a quantity of virus equivalent to 1–2 % of the intracellular virus was present in the supernatant medium at each assay. Concomitantly, the number of intracellular infectious units progressively decreased.

From 18 to 52 hr. sample monolayers were stained by Giemsa. Although the visible particles in the inclusions could not be counted accurately, they obviously exceeded the numbers estimated by infectivity titrations.

The eclipse phase of inclusion blennorrhoea virus at 30°. Inclusions could not be seen in infected monolayers after 24 and 48 hr. incubation at 30° suggesting that the maturation of inclusion blennorrhoea virus was affected. To ascertain whether the virus replicated during incubation at 30°, the technique for obtaining one-step growth curves was modified after adsorption at 30°, by continuing incubation of the washed monolayers at this temperature for a further 24 hr. Thereafter the temperature was raised to 37° and the intracellular infectious units assayed periodically. The number of infected cells did not decrease as a result of incubation at 30° indicating that the intracellular virus remained viable. The curve itself (Fig. 3) ran parallel to those obtained previously and the yield of sixty infectious units/inclusion was not affected (Fig. 2). After 24 hr. at 30°, the incubation period required for the production of infectious units at 37° was reduced by about 8 hr. (Fig. 3). Thus inclusion blennorrhoea virus in eclipse continued to mature at 30° at only one-third of the rate at 37°.

DISCUSSION

The replication of inclusion blennorrhoea virus in HeLa cells was studied by a technique similar to that reported previously for vaccinia virus (Furness & Youngner, 1959*a*, *b*) and poliovirus (Furness, 1961) in which multiplication in singly infected cells is synchronized by adsorbing virus at temperatures which retard the rate of replication. The rate of maturation of inclusion blennorrhoea virus at 30° was one-third that at 37°; it was therefore negligible during the 2 hr. adsorption period and growth of the virus was virtually synchronized.

Our results indicated that this agent behaved like other animal viruses in that it had a stage during which infective virus could not be demonstrated. Meningopneumonitis virus of the psittacosis group was grown in tissue cultures of allantoic membranes (Girardi, Allen & Sigel, 1952) and L cells (Higashi, Notake & Fukada, 1959) with similar results. After adsorption to the cells, inclusion blennorrhoea virus and meningo-pneumonitis virus were not detected for 20–23 hr. Thereafter they increased exponentially until a peak was reached at 34–38 hr. Both viruses were heat labile at 37° and 35°, respectively. Heat inactivation of inclusion blennorrhoea virus during adsorption was obviated by our techniques. No evidence, however, was presented to exclude this possibility during adsorption of meningo-pneumonitis virus so it is possible that the assays of this virus underestimated the total number of infectious units. Nor was the release of meningopneumonitis virus from infected cells reported. Infective inclusion blennorrhoea virus was found in the supernatant medium suggesting spontaneous release from the inclusions. Since inclusion blennorrhoea virus is labile at 37° the unexpectedly small amount of infective virus found in the supernatant medium could be accounted for by heat inactivation and adsorption to the monolayer.

There was no evidence of cell to cell spread of virus as occurred in similar experiments with vaccinia virus in tissue culture (Furness & Youngner, 1959*a*, *b*).

We wish to thank Miss Margaret Ham for her excellent technical assistance.

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An Inducible Amidase Produced by a Strain of *Pseudomonas aeruginosa*

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SUMMARY

A strain of *Pseudomonas aeruginosa* was obtained which was able to grow on acetamide or propionamide as sole source of carbon and nitrogen. When grown on these amides, whole bacteria and cell-free extracts rapidly hydrolysed acetamide, glycollamide, acrylamide and propionamide and slowly hydrolysed formamide and butyramide. *N*-Methylformamide, *N*-methylacetamide, *N*-ethylacetamide, *N*-acetylacetamide, *N*-methylpropionamide, *N*-ethylpropionamide, lactamide and methyl carbamate were found to be non-substrate inducers of the amidase when the organism was grown in succinate + ammonium chloride medium. *N*-Methylformamide, *N*-methylacetamide, lactamide and methyl carbamate did not inhibit propionamide hydrolysis by whole bacteria, but under the same conditions glycine amide, iodoacetamide and urea were effective inhibitors of amidase activity. *N*-Phenylacetamide, cyanoacetamide, glycine amide, sarcosine amide, β-hydroxypropionamide and thioacetamide were neither substrates nor inducers of the amidase in this strain, but inhibited amidase induction by *N*-methylacetamide in succinate + ammonium chloride medium. Formamide also inhibited amidase induction under the same conditions.

INTRODUCTION

Most of the studies on microbial amidases have been concerned with enzymes which attack glutamine (McIlwain, 1948; Hughes & Williamson, 1952), asparagine (Attenbern & Housewright, 1954) or nicotinamide (Oka, 1954; Halpern & Grossowicz, 1957). Amidases which attack the aliphatic amides formamide and acetamide have been reported by several workers in *Mycobacteria* spp. (Halpern & Grossowicz, 1957; Kimura, 1959; Pershin & Nesvad'ba, 1960). Lamaire & Brunel (1951) found an inducible cyanamidase in the yeast *Sterigmatocystis nigra* and Gorr & Wagner (1932, 1933) showed that *Torula utilis* was able to grow with acetamide or urea as the nitrogen source and that under these conditions an acetamidase was produced. No amidase was produced by this organism when the amide or urea was replaced by $(NH_4)_2SO_4$. Den Dooren de Jong (1926) reported that some *Pseudomonas* spp. could use aliphatic amides as the sole source of carbon and nitrogen, e.g. *Pseudomonas aminovorans* grew on propionamide, caproamide, lactamide or succinamide. Steiner (1959) found that a *Torulopsis* sp. used some aliphatic amides as a source of nitrogen, and that the dried organism could hydrolyse formamide, acetamide

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and benzamide. The amidase of *Torula utilis* (Gorr & Wagner, 1932) appears to have been an inducible enzyme, and it seems probable that the pseudomonads studied by Den Dooren de Jong (1936) also produced inducible amidases.

MATERIALS AND METHODS

Organism. Pseudomonas aeruginosa NCTC 8602 was obtained from the National Collection of Type Cultures. The strain used in this work has been designated 8602/A and was obtained from NCTC 8602 by the procedure described below. The stock culture of 8602/A was maintained in acetamide medium stored at 5° and subcultured at weekly intervals. Cultures were grown in 100 ml. conical flasks containing 50 ml. medium. The flasks were inoculated with 1 ml. of an 8 hr. acetamide-grown culture and during overnight growth in a water bath at 37° the cultures were shaken vigorously. Cultures were also grown overnight at 37° on Lemco agar contained in Roux bottles. Organisms were harvested at the end of growth by centrifugation at 10,000 *g* for 5 min. and washed once with distilled water.

Media. Lemco agar was prepared as described by Clarke & Meadow (1959). Minimal salt solution was modified from that described by Kogut & Podoski (1953): 1 g. KH_2PO_4 and 5 ml. trace element solution (see below) were made up to 995 ml. with distilled water after adjusting to pH 7·2 with N-NaOH. The solution was distributed in 100 ml. conical flasks and autoclaved at 115° for 10 min. and allowed to cool. Then 0·25 ml. cold 10 % (w/v) $MgSO_4.7H_2O$, sterilized in the same way, was added to each flask containing about 50 ml. medium. This procedure was adopted to avoid precipitation of $MgHPO_4$ which otherwise occurred during autoclaving. The minimal salt solution was stored at room temperature until required.

The trace element solution contained (mg./l.):
$FeSO_4(NH_4)_2SO_4.6H_2O$, 116; HBO_3, 232; $CoSO_4.7H_2O$, 95·6; $CuSO_4.5H_2O$, 8·0; $MnSO_4.4H_2O$, 8·0; $(NH_4)_6Mo_7O_{24}.4H_2O$, 22·0; $ZnSO_4.7H_2O$, 174.

Acetamide and propionamide media were made up as required by adding to the sterile minimal salt solution, sterile 8·0 M-acetamide or 4·0 M-propionamide to a final concentration of 0·08 M-amide. The amides were sterilized by Seitz filtration and were stored at 5°.

Succinate + ammonium chloride medium contained in 1 l.; sodium succinate. $6H_2O$, 13·0 g.; NH_4Cl, 1·1 g.; KH_2PO_4, 1·0 g.; $MgSO_4.7H_2O$, 0·5 g. and 5 ml. trace element solution. The medium was made up to 1 l. with distilled water, distributed in 100 ml. conical flasks and autoclaved at 115° for 10 min.

Preparation of cell-free extracts. Cell-free extracts of *Pseudomonas aeruginosa* strain 8602/A were prepared by passing 15–25 ml. of a suspension containing the equivalent of about 30 mg. dry wt. bacteria/ml. in 0·1 M-Sørenson's phosphate buffer (pH 7·2) through a bacterial press (modified after Milner, Lawrence & French, 1950) at a pressure of 5000 lb./sq.in. The apparaus was cooled to 5° before use. The exudate was collected in a centrifuge tube cooled in ice, and centrifuged at 10,000 *g* for 5 min. at 5° to sediment unbroken bacteria and large cellular debris. The supernatant fluid was stored at 5° and was used within 2 hr. of preparation. This procedure resulted in a high percentage of cell breakage, usually not less than 90 %.

Measurements of dry weights of bacteria. A standard curve relating optical density to dry weight was constructed with measured dilutions of a suspension of strain

3602/A. Measurements of optical density were made in an EEL (Evans Electroselenium Ltd.) colorimeter with a green filter OGR 1. The optical densities of suspensions of strain 8602/A were converted to dry weights by reference to this curve.

Protein estimation. Protein was estimated in cell-free extracts of strain 8602/A by the method of Lowry *et al.* (1951).

Estimation of amides. Amides were estimated colorimetrically by the formation of hydroxamates (Snell & Snell, 1954) by using an EEL colorimeter with green filter OGR 1. Standard curves were prepared for the amides estimated by this procedure; reference to these curves enabled a colorimeter reading to be converted to μg. amide/ml. This procedure estimated about 20 μg. acetamide/ml. and 40 μg. *N*-methylacetamide/ml.

Estimation of amidase activity. Hydrolysis of amides to produce ammonia was estimated by measuring the ammonia liberated by Conway's microdiffusion method (Conway, 1957). The assays were carried out in 25 ml. flasks containing a total volume of 5 ml. Bacterial suspensions, or cell-free extracts, in 0·1 M-Sørenson's phosphate buffer (pH 7·2), were shaken in a water bath at 37° for 10 min. to ensure temperature equilibration. The test amide, also at 37°, was then added to give a final concentration of 0·2 M-amide and four 1 ml. samples were removed at 5 min. intervals for ammonia estimations. No. 1 Conway units were used, containing 1 ml. of saturated K_2CO_3 solution in the outer chamber, and 1 ml. 1% (w/v) boric acid, with bromcresol green + methyl red indicator, in the centre well. The saturated carbonate solution served not only to release the ammonia but to stop amidase activity. The boric acid solution was titrated with 0·02 N-HCl after incubation for 2 hr. at room temperature. Enzyme and substrate blanks were included. Acetamide hydrolysis by whole bacteria was optimal at pH 7·2 with an amide concentration 0·2 M; these conditions were therefore used for amidase assays. Propionamide was later found to be the best substrate (Table 3) and was used as the standard amide to compare amidase activities of enzyme preparations produced under various conditions. Ammonia production from propionamide was linear over the 15 min. period of incubation and was a linear function of bacterial concentration up to about the equivalent of 1 mg. dry wt. bacteria/ml.

Hydrolysis of amides which did not produce ammonia (e.g. *N*-methylacetamide) was studied under the same conditions, but instead of estimating ammonia in the 1 ml. samples removed from the reaction flask, the amide remaining was measured by conversion to hydroxamate as described above.

Amides. A complete list of the amides used in this investigation is given in Table 6. The *N*-substituted amides, *N*-ethylformamide, *N*-ethylacetamide, *N*-methylpropionamide and *N*-ethylpropionamide were synthesized by the method of Galat & Elion (1943). *N*-Acetylacetamide was synthesized by the method of Titherley (1901). β-Hydroxypropionamide was prepared as described by Gresham *et al.* (1951). Pyruvamide was synthesized by the method of Anker (1948). Glycollamide was obtained by ammoniolysis of polyglycollide, and lactamide was prepared in a similar way from lactide. Dr B. R. Rabin of this department provided the glycine and sarcosine amides and the other amides used were the best available commercial preparations.

RESULTS

Development of pseudomonad strain 8602/A

Preliminary tests with a number of laboratory strains of *Pseudomonas* spp. showed that none of them grew in a minimal salt medium with 0·02 M-acetamide as sole source of carbon + nitrogen. *Pseudomonas aeruginosa* NCTC 8602 was retested in this medium with the addition of 0·2% (w/v) glucose and found to grow well. No pigment production was observed in the first subculture in this medium, but after three subcultures growth was accompanied by pigment production. This strain now grew on 0·02 M-acetamide or 0·02 M-propionamide without added glucose. This new strain was designated 8602/A to distinguish it from the parent strain NCTC 8602 which was unable to grow with 0·02 M-acetamide as sole source of carbon + nitrogen. It was found that optimum growth of strain 8602/A was obtained with 0·08 M-acetamide and an initial pH of 7·2; these conditions were used to produce amide-grown organisms.

Amidase induction in pseudomonad 8602/A

Amidase estimations were made on whole bacteria and cell-free extracts of *Pseudomonas aeruginosa* NCTC 8602 grown on Lemco agar and strain 8602/A grown on Lemco agar and in acetamide medium. The results given in Table 1 show that both strains, when grown on Lemco agar, had a very low activity with acetamide as substrate, but that whole bacteria and cell-free extracts of 8602/A grown on acetamide had much higher activities. The figures for whole bacteria are expressed per mg. dry wt. organism and those for cell-free extracts per mg. protein.

Table 1. *Amidase production by* Pseudomonas aeruginosa *NCTC* 8602 *and strain* 8602/*A*

Strain	Medium	μmole acetamide hydrolysed/100 min.	
		mg. dry wt. bacteria	mg. protein in cell extract
NCTC 8602	Lemco agar	12	6
8602/A	Lemco agar	13	6
	Acetamide medium	374	272

The Lowry method gave values for protein in the cell-free extracts which were too high, i.e. 1 mg. dry wt. bacteria gave apparently 1 mg. protein. There was always some loss of material during disruption of the bacteria and the amidase in the cell-free state was less stable than in unbroken bacterial cells, so that the figures for cell-free extracts cannot be compared directly with those for corresponding whole bacteria. The results do show however that the only cell-free extract with high amidase activity was that obtained from acetamide-grown bacteria of strain 8602/A, so that it could be concluded that the enzyme was induced during growth in acetamide medium.

Acetamide and propionamide as inducers and substrates

Acetamide and propionamide were tested as substrates of the amidase in whole bacteria and cell-free extracts of 8602/A grown on Lemco agar, or acetamide- or propionamide-media. Table 2 shows that acetamide-grown bacteria and cell-free extracts had the highest amidase activities and were about twice as active as propionamide-grown preparations. Propionamide was however a better substrate and was hydrolysed 2–2·5 times as fast as acetamide by all preparations. Propionamide was therefore used as the standard substrate for all subsequent amidase assays and strain 8602/A was grown in acetamide medium to produce bacteria with high amidase activity. It must be stressed that the figures given for enzyme activities represent the amidase present in the bacteria after the end of growth under the given conditions. Since both amides are substrates of the amidase they are metabolized during the growth period so that the concentration of amide which may act as inducer decreases to zero during growth. Much higher degrees of amidase activity were measured in acetamide-grown bacteria harvested 1–2 hr. before the end of the logarithmic growth period.

Table 2. *Amidase induction in* Pseudomonas aeruginosa *strain* 8602/*A by acetamide and propionamide*

	μmole acetamide hydrolysed/100 min./		μmole propionamide hydrolysed/100 min./	
Medium	mg. dry wt. bacteria	mg. protein in cell extract	mg. dry wt. bacteria	mg. protein in cell extract
Lemco agar	13	6	20	12
Acetamide medium	374	272	980	740
Propionamide medium	202	104	500	230

Table 3. *Substrate specificity of acetamide-grown* Pseudomonas aeruginosa *strain* 8602/*A*

	μmole amide hydrolysed/100 min./	
Amide (0·2 M)	mg. dry wt. bacteria	mg. protein in cell extract
Formamide	74	34
Acetamide	374	272
Propionamide	980	740
Butyramide	12	4
iso-Butyramide	0	0
Valeramide	0	0
Glycollamide	450	380
Acrylamide	480 (174)	360 (120)

Substrate specificity

Acetamide-grown bacteria were used to test some acetamide analogues as substrates of the enzyme; a complete list of all the amides tested is given in Table 6. The results given in Table 3 show that, besides acetamide and propionamide, only glycollamide and acrylamide were rapidly hydrolysed. Hydrolysis of acrylamide

was not linear with time, presumably due to inhibition of the enzyme by acrylamide or acrylic acid or both. The high initial rate could not be accounted for by the presence of acetamide or propionamide as impurities in the acrylamide preparation. The figure given in Table 3 for acrylamide hydrolysis is the rate over the first 5 min. and the rate of hydrolysis between 10 and 15 min. is given in parenthesis. Formamide and butyramide were slowly hydrolysed; the other amides tested were not hydrolysed at all.

Inducer specificity

Tests for amidase induction were carried out in the succinate + ammonium chloride medium so that the amides were not necessary as either carbon or nitrogen sources. The substrate amides, with the exception of formamide, were found to be enzyme inducers in this medium at 10^{-2}M, although glycollamide was a poor inducer. Acrylamide could not be tested as an inducer under these conditions since it inhibited growth at 10^{-3}M. Several non-substrate amides were also found to be effective inducers at 10^{-2}M (Table 4).

Table 4. *Amidase induction in* Pseudomonas aeruginosa *strain* 8602/*A grown in succinate + ammonium chloride medium*

Amide added	10^{-2}M	10^{-3}M
	μmole propionamide hydrolysed/ mg. dry wt. bacteria/100 min.	
None	4	4
Formamide	4	4
Acetamide	200	4
Propionamide	130	4
N-Methylformamide	760	4
N-Ethylformamide	18	4
N-Methylacetamide	1270	8
N-Ethylacetamide	200	4
N-Acetylacetamide	1400	28
N-Phenylacetamide	4	4
N-Dimethylacetamide	4	4
N-Methylpropionamide	300	4
N-Ethylpropionamide	150	4
Cyanoacetamide	4	4
Glycine amide	4	4
Sarcosine amide	4	4
Glycollamide	24	4
β-OH-Propionamide	4	4
Lactamide	1300	20
Fumaramide	4	4
Methyl carbamate	170	4
Urea	4	4
Thioacetamide	4	4

Each amide was tested at three concentrations (10^{-2}M, 10^{-3}M, 10^{-4}M). The amides were added as sterile M-preparations in distilled water to the flasks of succinate + ammonium chloride medium which were then inoculated with acetamide-grown 8602/A. After overnight growth, the amidase activities of washed suspensions were determined with propionamide as substrate. No appreciable induction was observed with any amide at 10^{-4}M or 10^{-3}M, but at 10^{-2}M, *N*-methylformamide, *N*-methylacetamide, *N*-acetylacetamide and lactamide induced high amidase

activity; appreciable degrees of amidase activity were induced by *N*-ethylacetamide, *N*-methylpropionamide, *N*-ethylpropionamide and methyl carbamate.

Figure 1 shows the amidase values at the end of growth with an amide concentration between 10^{-2}M and 10^{-3}M for *N*-methylacetamide, lactamide and acetamide. It can be seen from this figure and Table 4 that the non-substrate inducers *N*-methylacetamide and lactamide at 10^{-2}M induced six times as much amidase as was induced by acetamide under the same conditions.

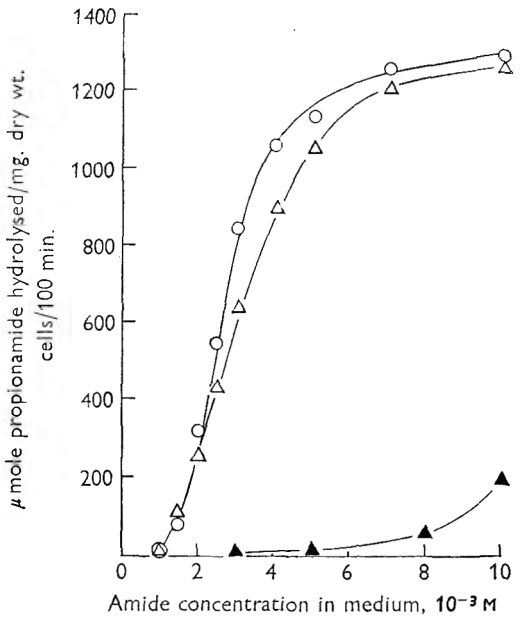

Fig. 1

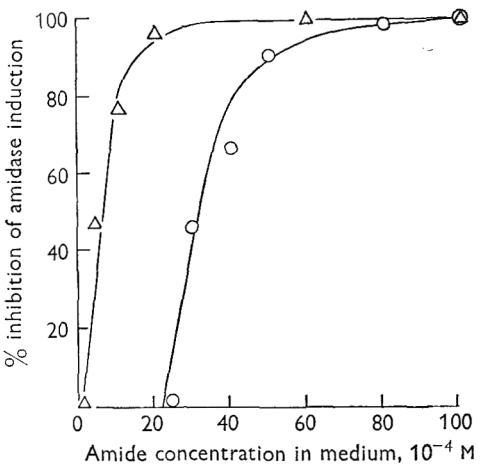

Fig. 2

Fig. 1. Effect of inducer concentration on amidase production by *Pseudomonas aeruginosa* strain 8602/A. Cultures grown in succinate + ammonium chloride medium with inducer amides added. -▲-▲- = acetamide; -△-△- = *N*-methylacetamide; -O-O- = lactamide.

Fig. 2. Effect of cyanoacetamide and formamide on amidase induction by *N*-methylacetamide in *Pseudomonas aeruginosa* strain 8602/A. Cultures grown in succinate + ammonium chloride medium with 10^{-2}M-*N*-methylacetamide added as inducer. -△-△- = cyanoacetamide; -O-O- = formamide.

Enzyme inhibition

Some of the non-substrate amides were tested as enzyme inhibitors, using acetamide-grown bacteria and propionamide as substrate. Propionamide hydrolysis by intact bacteria was not inhibited by the non-substrate inducers *N*-methylformamide, *N*-methylacetamide, lactamide and methyl carbamate (see Table 6). Glycine amide and urea, which were not inducers, inhibited propionamide hydrolysis by 85 and 100%, respectively, when present at the same concentration as propionamide (0·2M). Iodoacetamide at this concentration also inhibited hydrolysis completely. Thioacetamide, *N*-phenylacetamide and malonamide did not inhibit the amidase in intact bacteria.

Inhibition of enzyme induction

Since the substrate amides were hydrolysed during growth, tests for inhibition of enzyme induction were carried out with the non-substrate inducer *N*-methylacetamide in succinate + ammonium chloride medium. The inducer was added to 10^{-2}M final concentration and the amides tested as inhibitors of induction were added to the same concentration. The cultures were grown overnight and the amidase activities of washed suspensions of whole bacteria were then determined with propionamide as substrate; the results are given in Table 5. Figure 2 shows the effect of different concentrations of formamide and cyanoacetamide on amidase induction by 10^{-2}M *N*-methylacetamide. At 10^{-2}M both amides completely inhibited amidase induction, but whereas cyanoacetamide also produced complete inhibition at 10^{-3}M, formamide caused no inhibition at this concentration.

Table 5. *Effect of amides on amidase induction by* N-*methylacetamide in* Pseudomonas aeruginosa *strain* 8602/*A grown in succinate + ammonium chloride medium*

Amide added to medium containing 10^{-2}M *N*-methylacetamide	μmole propionamide hydrolysed/mg. dry wt. bacteria/100 min.	Inhibition (%)
None	1270	—
Formamide	4	100
N-Phenylacetamide	4	100
N-Dimethylacetamide	1260	0
Cyanoacetamide	4	100
Glycine amide	10	100
Sarcosine amide	110	92
β-OH-Propionamide	14	100
Thioacetamide	50	97

Comparison of amides as substrates, inducers and inhibitors

Table 6 lists all the amides used in this investigation and summarizes the results obtained. Figures for the substrate, inducer and inhibitor activity of these amides are given in Tables 3–5 and are represented in Table 6 by + signs to compare their relative activities. Substrate activity was measured with acetamide-grown bacteria; for all other estimations propionamide was the enzyme substrate. Induction experiments were carried out in succinate + ammonium chloride medium with 10^{-2}M test amide. Enzyme inhibition was measured with acetamide-grown bacteria and inhibition of induction in succinate + ammonium chloride medium with *N*-methylacetamide as inducer. All the amides were tested as substrates and inducers and from these results those amides were selected which could appropriately be tested for inhibition of enzyme activity or induction.

It can be seen (Table 6) that all the substrate amides had an unsubstituted amide group and that the most effective substrate was propionamide. The non-substrate inducers fell into two groups: (1) mono-*N*-substituted aliphatic amides in which the substituent group was methyl, ethyl or acetyl but not phenyl; (2) some amides with substituent groups on the carbon chain which could be considered as analogues of either acetamide or propionamide, i.e. methyl carbamate and lactamide.

Amides which inhibited enzyme action, or the induction of enzyme by *N*-methylacetamide, can also be considered to be acetamide or propionamide analogues; of these only glycine amide inhibited both enzyme activity and induction.

Table 6. *Comparison of amides as substrates, inducers and inhibitors of* Pseudomonas aeruginosa 8602/*A amidase*

Amide		Substrate	Inducer	Enzyme inhibitor	Induction inhibitor
Formamide	$HCONH_2$	+	−	.	+
Acetamide	CH_3CONH_2	++	++	.	.
Propionamide	$CH_3CH_2CONH_2$	+++	++	.	.
Butyramide	$CH_3CH_2CH_2CONH_2$	Tr.	−	.	.
iso-Butyramide	$(CH_3)_2CHCONH_2$	−	−	.	.
Valeramide	$CH_3(CH_2)_3CONH_2$	−	.	.	.
Hexamide	$CH_3(CH_2)_4CONH_2$	−	.	.	.
N-Methylformamide	$HCONHCH_3$	−	+++	−	.
N-Ethylformamide	$HCONHC_2H_5$	−	+	.	.
N-Methylacetamide	$CH_3CONHCH_3$	−	+++	−	.
N-Ethylacetamide	$CH_3CONHC_2H_5$	−	++	.	.
N-Acetylacetamide	$CH_3CONHCOCH_3$	−	+++	.	.
N-Phenylacetamide	$CH_3CONHC_6H_5$	−	−	−	+
N-Dimethylacetamide	$CH_3CON(CH_3)_2$	−	−	−	−
N-Methylpropionamide	$CH_3CH_2CONHCH_3$	−	++	.	.
N-Ethylpropionamide	$CH_3CH_2CONHC_2H_5$	−	++	.	.
Cyanoacetamide	$CH_2CNCONH_2$	−	−	.	+
Iodoacetamide	CH_2ICONH_2	−	.	+	.
Glycine amide	$CH_2NH_2CONH_2$	−	−	+	+
Sarcosine amide	$CH_2NH(CH_3)CONH_2$	−	−	−	+
Glycollamide	$CH_2OHCONH_2$	++	+	−	+
Acrylamide	$CH_2 = CHCONH_2$	++	.	+	.
β-OH-propionamide	$CH_2OHCH_2CONH_2$	−	−	−	+
Lactamide	$CH_3CHOHCONH_2$	−	+++	−	.
Pyruvamide	$CH_3COCONH_2$	−	.	.	.
Fumaramide	$CHCONH_2$ ‖ $CHCONH_2$	−	−	−	.
Malonamide	$CH_2(CONH_2)_2$	−	−	−	.
Methyl carbamate	CH_3OCONH_2	−	++	−	.
Urea	NH_2CONH_2	−	−	+	.
Benzamide	$C_6H_5CONH_2$	−	.	−	.
Thioacetamide	CH_3CSNH_2	−	−	−	+

+++, ++, +, Tr. = relative activities (see Tables 3–5); − = no activity; . = not tested.

DISCUSSION

The results in this paper confirm the implications of den Dooren de Jong's survey (1926) that some species of *Pseudomonas* are able to produce aliphatic amidases. The amidase produced by *P. aeruginosa* 8602/A is clearly an inducible enzyme differing in specificity from the acetamidase described in *Torulopsis* sp. (Steiner, 1959) and *Torula utilis* (Gorr & Wagner, 1932, 1933). The amidase of *Torulopsis* sp. also hydrolysed aromatic amides and the acetamidase of *Torula utilis* hydrolysed lactamide as well as acetamide. Although lactamide was not a substrate of the

P. aeruginosa 8602/A amidase, it could act as an inducer. The *Torula utilis* amidase was induced by growth on urea, which could also be used as the sole source of nitrogen for growth by this organism; but urea was neither a substrate nor an inducer of the *P. aeruginosa* 8602/A amidase.

It seems probable from the results obtained for substrate specificity and the relative values for propionamide and acetamide hydrolysis by bacteria grown in acetamide or propionamide medium, or with non-substrate inducers, that only one amidase was induced.

The ease with which the amidase-producing strain 8602/A was obtained from the parent strain *Pseudomonas aeruginosa* NCTC 8602 suggests that subcultivation on the glucose + acetamide medium may have selected organisms already capable of producing the enzyme. It is possible that other *Pseudomonas* spp. may also be induced to produce amidase, although the only strain tested so far (*P. fluorescens* KB_1) was not induced by *N*-methylacetamide in the succinate + ammonium chloride medium.

In considering the specificity of substrates and inducers of this amidase in more detail several factors emerge. Only aliphatic amides containing two or three carbon atoms were rapidly hydrolysed by bacteria or cell-free extracts: acetamide, glycollamide, acrylamide and propionamide were good substrates, but formamide with one carbon and butyramide with four carbons were only slowly hydrolysed. It is possible that hydrolysis of formamide and butyramide was due to other enzymes; examination of the specificity of the purified amidase should answer this question. It is impossible to estimate the relative substrate activity of acrylamide because hydrolysis of this amide was accompanied by inhibition, possibly due to the double bond present in both substrate and product. Glycollamide (hydroxyacetamide) was hydrolysed by whole bacteria and cell-free extracts more rapidly than acetamide, so that in this respect the hydroxyl group behaved as a partial substitute for the CH_2 group of propionamide which appears to make it a better substrate than acetamide. Glycollamide and propionamide may have the optimum size for the substrate molecule since lactamide, which has both an hydroxyl group and an extra CH_2 group, as compared with acetamide, was not a substrate.

Of the *N*-substituted amides, *N*-methylacetamide and *N*-acetylacetamide were the most effective inducers. This suggests a size limitation for both the substituent group and the whole inducer molecule, since *N*-ethylacetamide, *N*-methyl- and *N*-ethylpropionamide were much less effective as inducers. *N*-phenylacetamide, although unable to induce could still inhibit induction by *N*-methylacetamide, presumably by competition at a site in the bacterial cell having a specific role in amidase induction. It is interesting that *N*-methylformamide was an inducer whereas formamide itself was not an inducer, was a very poor substrate, and inhibited enzyme induction by *N*-methylacetamide. *N*-Dimethylacetamide, with two substituent groups on the amide nitrogen, was neither a substrate nor inducer, suggesting that at least one free amide hydrogen is required for induction while both are necessary for substrate activity.

Of the acetamide and propionamide analogues with substituents in the carbon chain, only two were substrates (glycollamide and acrylamide) and two more were non-substrate inducers. The hydroxy-substituted propionamides had different effects according to the position of the hydroxyl group; α-hydroxypropionamide

(lactamide) was as good an inducer as *N*-methylacetamide. The lactamide was prepared by a chemical synthesis so that it is not known at present whether the L and D forms are equally effective. β-Hydroxypropionamide (hydracrylamide) had no inducer activity and inhibited amidase induction by *N*-methylacetamide. Methyl carbamate was also an inducer and has a structural resemblance to acetamide, but it is apparent from its chemical properties that the amide group in this compound and in urea react differently from the amide group in acetamide. Methyl carbamate was not a very active inducer and had no effect on enzyme activity; urea inhibited the enzyme and was not an inducer.

Glycine and urea both inhibited enzyme action in acetamide-grown bacteria and it may be significant that these two compounds both contain an additional NH_2 group. Glycine amide was the only enzyme inhibitor which also inhibited enzyme induction by *N*-methylacetamide. The inhibition of enzyme activity by iodoacetamide may be due to its known reaction with thiol groups (Hellström, 1932) rather than to its structural resemblance to acetamide.

The alteration in the acetamide molecule of replacing the oxygen by sulphur to give thioacetamide was enough to destroy substrate and inducer properties, but thioacetamide inhibited enzyme induction although not enzyme activity in intact bacteria. In this respect it differs from the thiogalactosides, some of which can induce β-galactosidase in *Escherichia coli* (Monod, 1956).

These results are in general agreement with the conclusions reached by Monod (1956) from studies with β-galactoside analogues, that inducer action is a distinct and separate function from substrate action. With the exception of studies on substrate specificity, the experiments reported here were carried out with intact bacteria. This was inevitable in studying amidase induction in growing cultures but it does raise questions about permeability. It might be argued that compounds which were able to inhibit enzyme induction by *N*-methylacetamide were able to enter the bacterial cell, but this is not necessarily true, since it is not possible to define the point at which either the inducer or the inhibitor acts. Induction might be inhibited by competition at the point of entry into the bacterial cell or at a site within the cell. The high concentrations of inducer necessary (10^{-2} M) for maximum induction suggests that *Pseudomonas aeruginosa* 8602/A does not possess a mechanism for concentrating amides within the cell, unlike the β-galactoside permease described for *Escherichia coli* (Rickenberg, Cohen, Buttin & Monod, 1956). However, *Pseudomonas* spp. have been shown to possess inducible permeases for organic acids (Shilo & Stanier, 1957; Clarke & Meadow, 1959) and the permeability of these organisms to amides is worth investigation.

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The Growth-factor Requirements of *Haemophilus influenzae*

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SUMMARY

Fifteen strains of *Haemophilus influenzae* were found to require for growth, in addition to coenzyme I (diphosphopyridine nucleotide; DPN) and haematin, the following substances: pantothenic acid, thiamine, uracil. Some of the strains also required a purine, accepting xanthine, hypoxanthine or guanine, but not adenine. Cysteine (or glutathione) was also needed for luxuriant growth. A medium is described which yields crops of about 10^{10} organisms/ml. after incubation for 18 hr. at 34°. Sheep red cells, but not horse red cells, contain a DPN-ase, located in the stroma, which rapidly destroys any DPN added to them and also destroys the contained DPN when the cells are lysed.

INTRODUCTION

Davis (1917, 1921*a*, *b*) showed that two quite different growth factors are required for the growth of *Haemophilus influenzae*. These were later identified as haematin (Fildes, 1921; Lwoff & Lwoff, 1937*a*, *b*, *c*; Lwoff & Pirosky, 1937) and cozymase (Lwoff & Lwoff, 1937*a*, *b*). Although it was confirmed that the addition of cozymase (diphosphopyridine nucleotide; DPN) and haematin (10 μg./ml. each) to nutrient agar permitted the growth of *H. influenzae*, and that both factors were required, the addition of the two growth factors to a Casamino acids medium gave no growth even when supplemented with tryptophan and the ash from nutrient broth. By using the 'well' technique (see below), it was found that the addition of Tryptone (trypsin digested casein; Oxo brand) Stokes's yeast extract (Stokes, 1955), yeast extract dialysis diffusate (Cohen & Wheeler, 1946) or autoclaved yeast extract dialysis diffusate to the Casamino acids + DPN + haematin + agar basal medium all permitted good growth. It was further found that the addition of glucose (0·1 %, w/v) to the Casamino acids + DPN + haematin + yeast extract diffusate medium resulted in a five- to six-fold increase in yield. The addition of glucose to routine 'chocolate' agar medium, however, did not improve the yield. Treatment of the yeast extract dialysis diffusate with nitrous acid did not diminish its growth-promoting properties. Systematic investigations were therefore made to discover the growth-producing factors.

METHODS

Basal medium (g.): Casamino acids (Difco Technical), 1·2; NaCl, 0·3; Na glutamate, 0·15; cysteine HCl, 0·01; KH_2PO_4, 0·05; $MgCl_2.6H_2O$, 0·04; $CaCl_2.2H_2O$, 0·001; $FeSO_4.7H_2O$, 0·00125; $CuSO_4.5H_2O$, 0·000125; water to 100 ml. To this

mixture was added 5 % (w/v) granular charcoal (for gas-adsorption; British Drug Houses Ltd.), previously well washed with hot water to remove 'fines' and an appreciable amount of oily material. The medium was well stirred, filtered, adjusted to pH 7·6 with NaOH and 100 ml. volumes placed in 100 ml. (nominal) screw-cap bottles containing 1·75 g. powdered agar (Difco); the mixture was sterilized and the agar dissolved by autoclaving at 121° for 15 min. with the caps screwed tight.

DPN solution (1000 μg./ml.). To 100 ml. sterile water were added and dissolved 100 mg. DPN, the solution sterilized by filtration through a 0·7 μ Gradocol membrane and stored in the refrigerator.

Haematin solution. To 80 ml. distilled water were added 200 mg. Na_2CO_3 and 120 mg. haematin and the two completely dissolved. The solution was Seitz-filtered (6 cm. mat, grade S.B.) and the mat washed through with 20 ml. distilled water. This gave a solution of about 1 mg. haematin/ml. (nominal since the Seitz filter-mat adsorbed appreciable amounts of haematin). This solution was stored in the refrigerator.

Growth-factor solutions. These were prepared in sterile distilled water at 1 mg./ml., except biotin, cobalamine, coenzyme A and cytochrome C which were used at 50 μg./ml. and which were initially sterile.

The test plate. To 100 ml. of the melted agar basal medium, at 85–90°, were added: (i) 1 ml. (equiv. 1 mg.) DPN solution; (ii) 1 ml. (equiv. 1 mg.) haematin solution; (iii) 0·2 ml. of 50 % (w/v) glucose solution. About 30 ml. lots of this molten mixture were poured into sterile Petri dishes. A quantity of the medium was retained and held at 55–60°. When the plates had set, five holes, equally spaced at the periphery of the agar, were cut out by using a stainless-steel tube, diameter 6 mm.; when necessary the cylinders of agar were removed with a sterile platinum wire.

To 5 ml. of the retained basal molten agar medium + DPN + haematin + glucose, in a tube, was added 0·1 ml. of the growth factor solution under test (giving a final concentration 20 μg./ml.). The tube was placed in boiling water for 3 min. and then, with a sterile Pasteur pipette, sufficient mixture was poured in to fill one hole and allowed to set. Five different mixtures were used for each plate. A suspension of *Haemophilus influenzae*, taken from a 18 hr. heated blood agar plate, was made up in 1 % (w/v) neutral Casamino acids to a concentration of about 2×10^9 organisms/ml. Streaks of implant were made with a platinum loop from the centre of the plate radially to cross over the well of each test substance. The implants were dried and the plate incubated for 18 hr. at 34°. When the substance under test was the limiting factor for growth, its growth-promoting effect produced a line of heavy confluent growth over the 'well', extending centrally and diminishing to virtually no growth at the centre ('medium control' part) of the plate. Five different strains of *H. influenzae*, obtained from the Routine Bacteriology Department, St Mary's Hospital, were used at the beginning of the investigation and fifteen different strains were used to check the conclusions.

RESULTS

Initial attempt to identify the factor in yeast extract dialysis diffusate

In these experiments it was assumed that only one supplementary growth factor was being provided by the yeast extract dialysis diffusate (hereafter yeast extract diffusate). By using the 'well' technique the following forty-eight substances were

separately tested in the DPN + haematin + glucose augmented basal medium: adenine sulphate, adenosine-5-monophosphate, adenylic acid, β-alanine, alloxan, *p*-aminobenzoic acid, ascorbic acid, adenosine triphosphate, biotin, cobalamine (vitamin B_{12}), choline, coenzyme A, cocarboxylase, cysteine hydantoin, cytochrome C, cytosine, folic acid, fructose-1:6-diphosphate, fructose-6-phosphate, gluconic acid, glucose-1-phosphate, glucose-6-phosphate, glutamine, glutathione, glycerol, β-glycerophosphate, glyoxylic acid, guanine, meso-inositol, hypoxanthine, nicotinamide, nicotinic acid, oleic acid, pantothenic acid, phospho-enol-pyruvic acid, 3-phosphoglyceric acid, pimelic acid, pyridoxamine HCl, pyridoxal phosphate, riboflavin, thiamine HCl, thiamine monophosphate ester, lipoic acid, thymine, uracil, vitamin E, vitamin K, xanthine. Not one of these compounds had any significant growth-promoting effect; nor did haemolysed washed horse red cells or boiled horse red cells. A mixture of basal medium and yeast extract diffusate (20 %, v/v) was always used on each plate as a positive control and always showed the growth-promoting effect.

Identification of four supplementary growth factors required by Haemophilus influenzae

Since not one of the forty-eight substances listed above had any growth-promoting effect for *Haemophilus influenzae* on the DPN + haematin + glucose augmented basal medium, whereas the addition of yeast extract diffusate did, it was decided to make an 'artificial yeast extract' and test this, as it now appeared probable that more than one supplementary growth factor was required and that all were present in the yeast-extract diffusate. The artificial yeast extract mixture was prepared to contain the following twenty-eight substances all at 1 mg./ml. except for biotin and cobalamine which were at 50 μg./ml.: adenine sulphate, adenosine-5-monophosphate, β-alanine, alloxan, *p*-aminobenzoic acid, adenosine triphosphate, biotin, cobalamine, co-carboxylase, cytochrome C, cytosine, folic acid, glutamine, guanine, hypoxanthine, meso-inositol, L-leucine, lipoic acid, nicotinamide, pantothenic acid, pimelic acid, pyridoxamine HCl, riboflavin, thiamine HCl, thiamine monophosphate ester, thymine, uracil, xanthine.

This mixture, diluted 1/50, promoted the growth of *Haemophilus influenzae* on the deficient medium more strongly than 20 % (v/v) of yeast extract diffusate. This confirmed the idea that two or more supplementary growth factors were required. The identification of the individual growth factors in the mixture was carried out as follows. To a molten sample of the basal medium were added two substances, e.g. $a+b$, and the mixture tested; to this mixture was then added substance c and the new mixture tested; to this mixture was added substance d and so on until the final mixture contained all the twenty-eight substances. From the results obtained a new 'complete' mixture was obtained containing fewer substances than the original twenty-eight as well as one identified growth factor. The latter was mixed with substance a to become a new first mixture, and the whole process repeated. For the next series the first mixture became the two identified growth factors only and the second mixture, these two substance $+a$ and so on.

In point of fact the analysis was not as straightforward as expected, for two reasons: (i) not all strains tested had the same requirements; (ii) there were alternative, but chemically related, substances which gave the same growth-promoting

effect. This was shown by another type of experiment, the 'one-missing' technique, namely, testing mixtures of the twenty-eight substances in which one of the twenty-eight substances was omitted in each case. Two substances were found to be absolute requirements for all strains, namely, pantothenic acid and uracil (uracil not replaceable by cytosine or thymine). The third growth factor was found to be thiamine, which was replaceable by co-carboxylase or thiamine monophosphoric ester. The fourth factor was a purine, not required by all strains. Of the four nucleotide purines tested, adenine had no growth promoting effect, but guanine, xanthine and hypoxanthine were interchangeable, with hypoxanthine the most effective. Uric acid had no growth promoting effect for purine-dependent strains planted on a purine-deficient medium. The addition of cysteine (or glutathione) to the Casamino acids basal medium was essential for growth. From these experiments a satisfactory medium was developed and may be prepared as follows.

Stock solution 1. 1 % (w/v) DPN. To 100 ml. distilled water is added and dissolved 1 g. 95 % DPN (Light and Co., Colnbrook, Bucks., England, or C. F. Boehringer & Söhne, Mannheim, W. Germany) and sterilized by filtration through a Gradacol membrane (0·7 μ, mean pore diameter) and stored in the refrigerator.

Stock solution 2. 0·5 % Haematin in a solution of 1 % (w/v) Na_2CO_3 + 5 % (w/v) $NaHCO_3$. To 180 ml. distilled water is added 2 g. Na_2CO_3 + 1·1 g. haematin, and complete solution facilitated by gently warming; in this solution is then dissolved 10 g. $NaHCO_3$. The solution is sterilized by Seitz-filtration, and the mat washed through with 20 ml. distilled water. The excess 10 % (w/v) haematin is added to compensate for its marked adsorption by the filter mat. This solution is stored in the refrigerator and protected from light.

Stock solution 3. 50 % (w/v) Glucose solution. A 50 % (w/v) solution of glucose in distilled water, sterilized by autoclaving at 121° for 15 min.

Stock solution 4. Uracil, hypoxanthine and cysteine HCl solution. To 200 ml. 0·1 N-HCl is added 1 g. uracil + 1 g. hypoxanthine + 10 g. cysteine HCl, and solution facilitated by gently warming.

The complete medium. To 1 l. distilled water is added 25 g. Oxoid no. 2 dehydrated nutrient broth (this provides an adequate supply of thiamine and pantothenic acid) and to this is added 2 ml. of stock solution 4 and 15 g. Davis agar (Davis Gelatine Co. Ltd., Warwick). The mixture is autoclaved in 1 l. bottle for 15 min. at 121°. After autoclaving and while the medium is still hot are added: 1 ml. stock solution 1, 2 ml. stock solution 2, 2 ml. stock solution 3. The medium is well mixed and dispensed into 30 to 35 Petri dishes (9 cm. diam.). This complete medium contains per ml.: 10 μg. each DPN, haematin, uracil, hypoxanthine; 100 μg. cysteine HCl; 1 mg. glucose; about 0·7 mg. $NaHCO_3$.

The harvest from an overnight culture (34°) on this medium is about 10^{10} organisms/ml. medium, which is about five times the yield from a chocolate agar plate. The organisms are uniformly minute coccobacilli.

Supplementary observations

Enriched blood-agar plate. The above complete medium may be used as the nutrient agar base for a blood plate, but only horse blood should be used (see below). When this especially enriched blood plate is used in routine bacteriology the colonial appearance of most microbes is different from usual. Not only does *Haemophilus*

influenzae grow freely but all pneumococci and streptococci grow exceptionally profusely, although with some alteration in their usual haemolytic characteristics. Thus: (i) β-haemolytic streptococci show greatly decreased haemolysis; (ii) 'viridans' streptococci show a narrower zone of green but a deeper green below the colony; (iii) pneumococci show almost no green zone, but cause the medium to appear almost black below the colony; the 18 hr. colonies are domed, not umbilicated, are very smooth and glistening, with a marked 'wet' appearance.

The chocolate agar plate and Haemophilus influenzae

It is common experience that whereas *Haemophilus influenzae* grows very poorly on a blood agar plate it grows luxuriantly when the same medium has been rapidly heated to 80–100°. More precisely, the growth of *H. influenzae* is poor on horse blood agar, very poor on human blood agar and does not occur on sheep blood agar; all become apparently equally good after heating. The fact that the organism grew luxuriantly on the heated blood medium showed that this contained all the necessary growth substances in the unheated state. The problem was therefore to account for the very greatly diminished growth on the unheated medium. The growth of *H. influenzae* on nutrient agar supplemented with 10 μg./ml. each of DPN and haematin was equal to that on a control chocolate agar plate. The omission of either growth factor from the nutrient agar prevented growth. That the failure of *H. influenzae* to grow on blood agar was not due solely to the two growth factors merely being inaccessible by being locked-up in the red cell was shown by culturing the organism on: (*a*) sheep blood agar medium in which the red cells had been lysed by distilled water or by saponin; (*b*) sheep blood agar to which had been added 10 μg./ml. each of pure haematin and DPN. In neither case did the *H. influenzae* grow. The possibility of growth inhibition by the serum moiety of the blood was excluded, since the same results were obtained when washed red cells were substituted for whole blood. On the other hand, it was found that the incorporation of heated washed sheep red cells in the nutrient agar base permitted luxuriant growth. These latter experiments suggested that it was not the sheep serum that prevented growth, and that the red cells were the source of both growth factors despite the absence of growth in the presence of intact or lysed red cells.

The yeast cell (a rich source of DPN) contains a DPN-pyrophosphatase, and in the extraction of DPN from yeast it is necessary to heat the cells rapidly to 80–100° to destroy this enzyme and thereby protect the simultaneously liberated coenzyme from hydrolysis (Tauber, 1949). From this it seemed probable that sheep red cells might contain a pyrophosphatase or diphosphopyridine nucleosidase (DPNase). That this is so was shown by incubating washed sheep red cells with pure DPN; the supernatant fluid from this mixture failed completely to permit growth of *H. influenzae* when added to a DPN-deficient medium.

In contrast to these findings with sheep blood agar the addition of haematin and DPN to a horse-blood agar plate permitted luxuriant growth of *Haemophilus influenzae*, and washed horse red cells did not inactivate DPN. *H. influenzae* grew well on a routine horse blood agar plate when the red cells had been haemolysed with saponin. From all this it is concluded that sheep red cells, but not horse red cells, contain a DPNase. By lysing washed sheep red cells with distilled water and

separating, by centrifugation, the stroma from the released haemoglobin, it was found that the stroma destroyed the DPN but that the stroma-free haemoglobin solution did not.

DISCUSSION

Two points arising from the above observations may be discussed: (*a*) the success experienced in the past on the growth of *Haemophilus influenzae* by workers using only peptone + DPN + haematin; (*b*) the type of DPN-splitting enzyme present in the sheep red cell stroma. (*a*) The Tryptone (a tryptic digest of casein) used in the preliminary work described above afforded moderate growth when added to the Casamino acids basal medium; from this it would seem that the enzyme preparation used to make the digest contained sufficient uracil, hypoxanthine, thiamine and pantothenic acid to permit moderate growth. Peptone, because of its meat origin, would contain more of all these growth factors.

(*b*) There are two types of DPN-splitting enzymes, a pyrophosphatase which hydrolyses the molecule to give nicotinamide mononucleotide and adenylic acid, and a nucleosidase (DPNase) which splits off nicotinamide. Gingrich & Schlenk (1944), using a peptone medium, found that nicotinamide riboside showed a V-factor activity for *H. influenzae* but was less efficient than DPN. Since the effect of the DPN-splitting enzyme in the sheep red cell stroma abolished the growth-promoting effect of a considerable excess of DPN it is thought probable that the enzyme in the stroma is of the nucleosidase type (DPNase) as was found by Hofmann & Rapoport (1955) to be the case in rabbit red cell stroma.

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The Culture of *Bordetella pertussis*

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SUMMARY

Bordetella pertussis grew profusely and retained its normal minute coccobacillary form when grown under 0·4 mm. of fluid lying on a Cohen & Wheeler agar medium + charcoal. Microbes grown in this way were fully antigenic in terms of the mouse protection test. It was easy to obtain harvests of $1{\cdot}2 \times 10^{11}$ organisms/ml.

INTRODUCTION

Apart from the classical work of Bordet and the introduction of the Bordet–Gengou medium for *Bordetella pertussis*, the most important observations concerning the growth conditions for this organism would seem to be those made by Pollock (1949) who demonstrated the growth-inhibitory effect of small quantities of unsaturated fatty acids in the medium. The incorporation of powdered charcoal, by Powell, Culbertson & Ensminger (1951), in agar-solidified Cohen & Wheeler (1946) medium presumably aids growth by absorbing such lipid inhibitors. The first modification to the Cohen & Wheeler agar medium + charcoal made in these laboratories was the substitution of washed granular charcoal (0·5 %, w/v, British Drug Houses Ltd. for gas adsorption) for the powdered charcoal. This modification yielded a charcoal-free white vaccine. The second modification arose from the observation that, when the volume of implant was excessive and the surface of the agar medium not truly horizontal, the growth of the organism was very poor where the medium surface was relatively drier than elsewhere, and also where the depth of fluid was excessive, but that there was an intermediate narrow zone of heavy growth where the surface was thinly covered with fluid. This observation was investigated.

METHODS

A setting table and the incubator shelves were adjusted to be truly horizontal. Flat 1-l. bottles containing 100 ml. of the agar medium were used, giving 200 sq. cm. of surface and 0·5 cm. depth of medium. Several of these bottles were seeded with different volumes of implant, of the same count, ranging from 5 to 20 ml./bottle and the agar surfaces were completely covered. Counts were made by opacity using the N.I.H. Standard Opacity tube, and antigenic potency by using the mouse intracerebral challenge method (W.H.O. Tech. Rep. Series, no. 61).

RESULTS

The harvest after growth on the above medium at 34° for 3 days from the 5 and 10 ml./200 sq.cm. of surface was $4{\cdot}5 \times 10^{10}$ organisms/ml. medium, compared with $3{\cdot}6 \times 10^{10}$ from the 15 ml. implant and $2{\cdot}7 \times 10^{10}$ from the 20 ml. implant. The volume chosen for routine use was 8 ml./200 sq.cm. of surface, giving a depth of fluid on the medium of 0·4 mm. Different strains of *Bordetella pertussis* differed in the yield given on this medium when grown in this way. The harvest, however, was rarely less than 3×10^{10} organisms/ml. medium, which means that when the culture was collected in 20–25 ml. saline the count (by opacity) was about $1{\cdot}2 \times 10^{11}$ organisms/ml. The same agar base could be used three times by adding to the agar residue (after harvesting) 10 ml. of 10 % (w/v) neutralized Casamino acids and re-incubating. This could be done on two successive occasions.

The organisms when grown in this way were extremely small and capsulated; large and filamentous forms were rare. Large and filamentous forms did appear, although not in large numbers, when the depth of fluid was too great, or when the yeast extract diffusate was omitted from the medium. In contrast to this it was found that when the same medium was used in Petri dishes, with a dry surface, growth was much slower than on a control Bordet–Gengou plate. After 3 days of incubation no colonies were visible on the Cohen & Wheeler (1946) medium + charcoal although well developed on the Bordet–Gengou plate. After a further 5 days of incubation, however, the same number of colonies were present on the Cohen & Wheeler medium + charcoal plates as were on the Bordet–Gengou plates for the same size of implant. Vaccines prepared from our selected strains grown in this modified Cohen & Wheeler agar medium + charcoal had an antigenic potency (by mouse intracerebral challenge), significantly greater than the British Standard pertussis vaccine, and agglutinated to 1/500 using Standard *Bordetella pertussis* Agglutinating Serum (Public Health Laboratory Service, Colindale). Apart from the ease of preparation of the medium, an important advantage of the method is that the cultures can be grown and harvested without mechanical aids (e.g. shakers for culture or centrifugation of crops).

DISCUSSION

The reason for the success of the technique described is thought to be that the inhibitors left in the medium normally concentrate at the agar surface in the form of soaps and free fatty acids and are therefore at their greatest concentration exactly where the implant is made. By covering the agar surface with a film of fluid, the inhibitors are transferred to the upper surface of the fluid, and the microbes are able to grow away from them, at the agar/fluid interface. And because *Bordetella pertussis* is an obligate aerobe, the thickness of the fluid film is critical. The ease with which *B. pertussis* grows when planted away from a free surface in the above way, the fact that the same agar base may be used three times and that the yeast extract diffusate may be omitted from the medium would together seem to indicate that the microbe has no essential growth factor requirements. Nevertheless, since it was found that the omission of yeast-extract diffusate, or too deep a film of fluid on the agar base, gave rise to the rapid appearance of large and filamentous forms, it would seem that a factor is required for normal growth under conditions of lowered oxygen tension.

The relative slowness of growth on the Cohen & Wheeler charcoal agar + charcoal medium as compared with that on a Bordet–Gengou plate, or when the base is covered by a thin film of fluid, would seem to indicate that the inhibitors in the medium act solely by increasing the mean generation time of the organism.

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The Culture of *Streptococcus pneumoniae*

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SUMMARY

In the fluid culture of *Streptococcus pneumoniae* in glucose + serum broth the part played by the serum appeared to be solely the regulation of pH value, and may be substituted by a suitable amount of sodium carbonate. The reason for the autolysis experienced in the fluid culture of pneumococci would appear to be due to hydrogen peroxide production. The addition of cysteine to a glucose + carbonate broth precluded the autolytic effect either by the cysteine giving an anaerobic condition of growth or by destroying H_2O_2.

INTRODUCTION

In the growth of pneumococci in fluid media it is common practice to use Wright's broth (Wright, 1933) or its equivalent, supplemented with serum and glucose. This medium has two disadvantages: (*a*) the serum is expensive and contaminates the microbes with antigenic foreign protein; (*b*) the incubation of the culture has to be closely watched since at a certain stage of growth marked autolysis takes place. This presents two problems: what part is played by the serum in the culture medium and what is the reason for the autolysis?

METHODS

Pneumococci, Types I, II and III were grown in modifications of the classical Wright's broth + serum + glucose medium. Note was taken of the changes in pH value and of the effect of the addition of H_2O_2 on the counts in cultures made in these different media.

RESULTS

The serum effect

When serum was omitted from the supplemented Wright's broth, growth began but soon ceased; the addition of serum, after the apparent cessation of growth, resulted in a marked increase in count. At the stage of apparent cessation of growth the medium was found to be at about pH 4, and that the addition of the usual 10 % (v/v) of serum changed the reaction to about pH 7, when growth began again. It seemed, therefore, that the part played by the serum might be no more than to control the reaction of the medium. This was tested as follows. Volumes (100 ml.) of 1 % (w/v) Casamino acids + 0·02 % (w/v) tryptophan + 5 % (v/v) yeast extract (Stokes, 1955) were seeded with Types I, II and III pneumococci in closed 100 ml.

bottles and incubated for 18 hr. at 34°. In no case was the growth heavy. To each bottle of medium was then added 1 ml. sterile 10 % (w/v) glucose and incubation continued for 7 hr. The growth increased appreciably and the cultures became acid again (about pH 4) and required 2 ml. of 3 % (w/v) Na_2CO_3 solution for neutralization. The neutralized cultures were incubated for a further 18 hr. The amount of growth again increased. Again the cultures became acid and required a second 2 ml. of 3 % (w/v) Na_2CO_3 solution for neutralization. These observations were followed up, using Wright's broth, where the main consideration was to observe the effect of pH control on the count. Samples (100 ml.) of broth were prepared as follows:

(*a*) Control; Wright's broth with no modification.

(*b*) Wright's broth + 1 ml. 10 % (w/v) glucose.

(*c*) Wright's broth + 1 ml. 10 % (w/v) glucose + 0·5 ml. 18·5 % (w/v) Na_2CO_3.

(*d*) (*c*) + 0·2 ml. stock solution 4 as used for the culture of *Haemophilus influenzae* (Holt, 1961; uracil, hypoxanthine, cysteine).

(*e*) (*c*) + 0·1 ml. normal horse blood (as source of catalase).

All bottles were seeded with Type I pneumococcus and incubated for 18 hr. at 34° with the results shown in Table 1.

Autolysis. Pneumococci are thought by some workers to produce peroxide during growth, the peroxide being responsible for the green coloration surrounding colonies on blood agar. It seemed that peroxide production might also be related to the autolysis which takes place in fluid culture since this occurs only after incubation for some hours. On blood agar cultures the young colony is domed and only later assumes the typical draughtsman appearance, and at a time when the growing microbes are spatially more removed from any protective action afforded by the embedded red cells. This possibility was investigated. Eight 100 ml. bottles of Wright's broth were seeded with Pneumococcus Type III and incubated overnight at 34°; the growth was poor. To these bottles the following additions were made:

Bottle no.	Addition	Relative opacity after 3 hr. further incubation
1	None (primary control)	+ +
2	+ 5 ml. '20 vols.' H_2O_2	+
3	+ 0·1 % (w/v) glucose	+ + +
4	+ glucose and peroxide	+
5	+ 5 ml. horse serum	+ + +
6	+ serum and peroxide	Almost clear
7	+ serum and glucose	+ + +
8	+ serum, glucose and peroxide	+

The bottles were then incubated a further 3 hr. with the results shown above.

These observations were confirmed with Pneumococcus Types I and II and extended in order to exclude the possibility that the hydrogen peroxide only prevented growth and did not simply have a direct lytic effect. Three bottles of Wright's broth were seeded with Pneumococcus Type II and incubated at 34° overnight. Next morning to two of the three bottles were added 10 ml. horse serum and 0·2 ml. 50 % (w/v) glucose; incubation was continued for 3 hr. The growth in the reinforced broth increased appreciably whereas there was no apparent change in the control.

At this stage 5 ml. of '20 vols.' H_2O_2 were added to one of the broth + serum + glucose mixtures and incubation continued for a further 3 hr. By this time the bottle containing added peroxide was less opaque than after incubation for 3 hr., indeed less than the control; whereas the culture containing serum + glucose without peroxide had increased considerably in opacity. It had already been noted, when working on the part played by serum, that the addition of solution 4 (Holt, 1962) which contained cysteine, to the medium resulted in good growth (see above). Cysteine might have a protective action against the effect of peroxide in two ways: (*a*) by making the medium anaerobic, in which circumstance peroxide would not be formed; (*b*) by its ready oxidation to cystine by any peroxide that might develop, then

Table 1. *Influence of* pH *control on count* (*by opacity*) *of* Streptococcus pneumoniae *grown in Wright's broth*

Pneumococcus Type I incubation 18 hr. at 34°

Medium	pH value after 18 hr.	Count (million/ml.)
(*a*) Control	7	About 300
(*b*) +0·1 % (w/v) glucose	4	1900
(*c*) +0·1 % (w/v) glucose +0·09 % (w/v) carbonate	7	1000
(*d*) +glucose+carbonate and 0·2 % (v/v) stock solution 4	6·5	2000
(*e*) +glucose+carbonate+0·1 % (v/v) blood	6·5	About 2500

Table 2. *Components of a medium for the culture of* Streptococcus pneumoniae

(1) Basal medium	
NaCl	2·5 g.
KH_2PO_4	0·5 g.
$MgSO_4.7H_2O$	0·4 g.
$CaCl_2$ (dried)	1 ml. 1 % (w/v) soln.
Proteose peptone (Difco)	20 g.
L-Cysteine HCl	0·15 g.
Yeast extract (Difco)	5 g.
Distilled water	1000 ml.
Adjusted to	pH 7·2
(2) 50 % (w/v) glucose in distilled water	
(3) 20 % (w/v) Na_2CO_3 solution	

decomposing the latter. The appearance of the colonies of pneumococci when grown on the especially enriched blood plate (Holt, 1962) which contains cysteine, is in keeping with this interpretation; the colonies were not umbilicated and the green halo was virtually absent although grown under fully aerobic conditions.

These two findings, namely the substitution of sodium carbonate for serum and the protective action of cysteine against autolysis, were adopted to give an excellent cultural technique as follows. The medium finally adopted is given in Table 2.

The basal medium was brought to the boil and filtered, and dispensed in 100 ml. and 1 l. volumes in screw-cap bottles. These and the glucose and carbonate solutions were sterilized by autoclaving at 121° for 15 min. Litre volumes of the basal

medium were placed in the incubator (34°) and seeded next morning with 100 ml. of overnight culture in the same medium; at the same time 2 ml. of the sterile 50 % (w/v) glucose was added. Incubation was continued for 2 hr. and then 5 ml. of 20 % (w/v) Na_2CO_3 solution added; incubation was continued for a further 5 hr.

The yield of pneumococci under these conditions was about 2000 million/ml. for Types I and III and about 3000 million for Type II. Incubation may be continued for a further 18 hr. without evidence of autolysis. Haemolytic streptococci also grew more profusely in this medium than in normal glucose broth.

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The Fate of Mesoinositol during the Growth of an Inositol-dependent Yeast *Kloeckera brevis*

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SUMMARY

An inositol-dependent yeast *Kloeckera brevis* was grown in a medium limited in inositol and the fate of the inositol determined. All of the inositol was taken in by the yeast and combined in an organic form. Fractionation of the yeast after disruption in a Hughes press showed that 31–38 % of the combined inositol was soluble in trichloroacetic acid solution and 43–65 % was soluble in neutral or acidified lipid solvents. The trichloroacetic acid extract contained three inositol components; one minor one was identified as inositol monophosphate. The major component was a neutral derivative of mesoinositol which was not identified as any known naturally-occurring form of combined inositol. The lipid extracted with neutral solvents contained an inositide whose properties were those of phosphatidyl inositol. The lipid inositol extracted with acidified solvents appeared to be present in a lipoprotein.

INTRODUCTION

It is now well established that many yeasts require inositol as an essential growth factor. So far attempts to demonstrate a metabolic lesion in such organisms caused by a lack of inositol have not been successful. It seemed likely therefore that a lead on the biochemical role of inositol in yeast growth might be provided by a study of the chemical fate of the inositol during growth. Yarbrough & Clark (1957) found that most of the inositol in *Schizosaccharomyces pombe* could be extracted with lipid solvents, e.g. chloroform + methanol mixtures, while Smith (1951) reported that with *Saccharomyces carlsbergensis* much was bound as a 'phytin-like' compound. Ridgway & Douglas (1958) showed that in *S. carlsbergensis* most of the combined inositol was present in the cytoplasmic particles rather than in the cell wall: about 40 % was soluble in lipid solvents and the remainder was not extracted with trichloroacetic acid solution.

In the present work *Kloeckera brevis* was used as the test organism. It has an absolute and highly specific dependence on mesoinositol for its growth (Campling & Nixon, 1954; Hartree, 1957). The fate of inositol in the growth medium was determined not only by direct analysis of the yeast fractions, but also by using ^{3}H-labelled inositol as a marker.

METHODS

Growth of Kloeckera brevis. A stock culture of *Kloeckera brevis* (NCYC 328) was maintained on agar slopes. The yeast was grown at 25° for 3 days in 250 ml. of the medium described by Campling & Nixon (1954) contained in large Erlenmeyer flasks. The cultures were shaken once daily. Meso-inositol was added to a concentration of 1 μg./ml.

Fractionation of yeast. The yeast was harvested by centrifuging (1000 *g* av. for 20 min.) and washed twice with water (1/5th volume of original culture); this resulted in a negligible loss of soluble inositol. The yeast was then either fractionated directly or after disruption in a Hughes (1951) press, with two pressings either at $-20°$ or $-78°$. A flow sheet of the fractionation is shown in Fig. 1. The yeast recovered from 1 l. of medium was extracted twice with 15 ml. warm $CHCl_3 + CH_3OH$ (1+1; v/v) for 10 min. and then once with 15 ml. of warm $CHCl_3 + CH_3OH$ (1+2; v/v). The supernatant fluids obtained by centrifuging were mixed and $CHCl_3$ added until the final solvent composition was $CHCl_3 + CH_3OH$ (2+1; v/v). The lipid extract was then washed with 0·2 volumes of either 0·9 % (w/v) NaCl or water to remove water-soluble non-lipid impurities according to Folch, Lees & Sloane-Stanley (1957). The washings, which contained some water-soluble combined inositol, were saved for adding to the trichloroacetic acid extract of the yeast.

The solvent-extracted yeast residue was then extracted twice with 15 ml. of 5 % (w/v) trichloroacetic acid solution for 5 min. at 4°. The extracts were combined with the washings from the lipid purification and the mixture then washed three times with equal volumes of ether to remove trichloroacetic acid. The aqueous layer was filtered to yield a clear water-soluble fraction.

The solvent and trichloroacetic acid extracted yeast residue was then extracted twice with 15 ml. $CHCL_3 + CH_3OH$ (2+1; v/v) containing 0·33 ml. concentrated HCl/100 ml., with the intention of extracting protein-bound lipid-inositol (see Lebaron & Folch, 1956).

Inositol assay. Inositol was assayed by the procedure of Campling & Nixon (1954) with *Kloeckera brevis* as the test organism. Combined inositol was hydrolysed for 18 hr. at 105° in 5 N-HCl, the acid removed *in vacuo*, the residue made up to a known volume with water and adjusted to pH 5 with NaOH before assay.

Radioactivity experiments. Inositol randomly-labelled with tritium (^3H) was obtained from Tracerlab Inc., Waltham, Mass., U.S.A., and had been prepared by the Wilzbach (1957) method. It was purified by two crystallizations from ethanol + water and the radioactivity was chromatographically homogeneous when tested on paper in several solvent systems. The activity was 7·8 μC/μmole. ^3H was assayed in a Nuclear Chicago (Chicago, U.S.A.) flow counter. Paper chromatograms were cut into 1 cm. wide strips before assay.

^{32}P-labelled yeast was prepared by growing *Kloeckera brevis* in Campling & Nixon's (1954) growth medium in which the concentration of KH_2PO_4 had been decreased to 0·3 g./l. and $H_3{}^{32}PO_4$ added at an activity of 2 μC/ml.

Paper chromatography and ionophoresis. Whatman No. 1 paper was used after washing with 2 N-acetic acid. Descending chromatography was carried out with the following solvents: (*a*) isopropanol + acetic acid + water (3+1+1; v/v); (*b*) ethyl acetate + propanol + water (13+24+7; v/v), and ascending with ethanol + 27 %

(w/v) NH_3 solution (3+2; v/v). High voltage (50 V/cm.) ionophoresis was carried out under toluene in an apparatus similar to that described by Ryle, Sanger, Smith & Kitai (1955) with a volatile pyridine + acetic acid buffer (pH 3·6). For preparative paper-electrophoresis or ionophoresis the fractions were run as strips; after location of the separated bands they were eluted by descending chromatography with water as solvent.

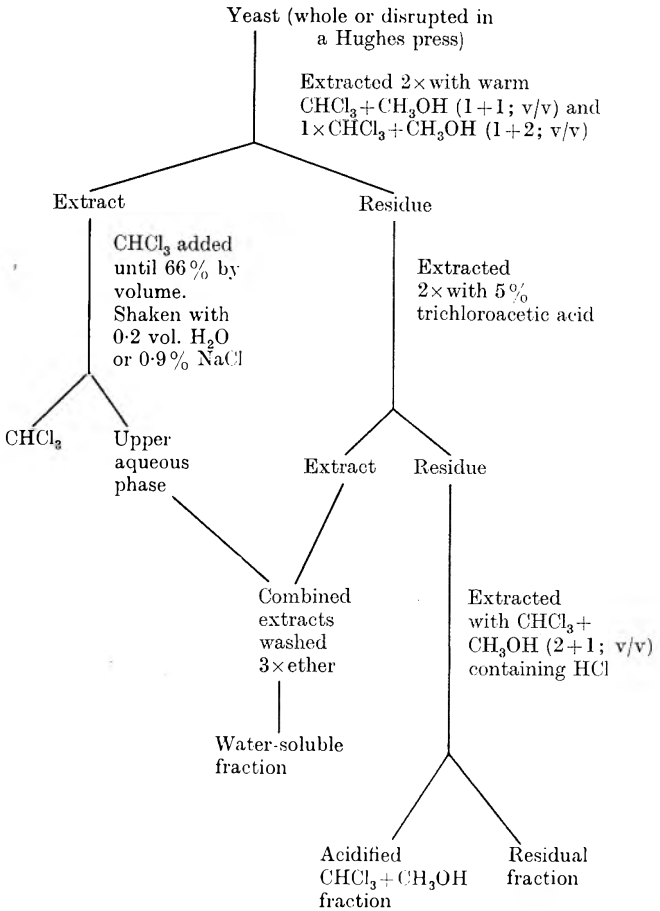

Fig. 1. Flow sheet of the fractionation of *K. brevis* cells.

Polyols were detected on paper by using the acetone + $AgNO_3$ and ethanolic NaOH reagents of Trevelyan, Procter & Harrison (1950); phosphate esters were detected with the acid molybdate reagent of Hanes & Isherwood (1949) followed by ultraviolet irradiation.

Materials. Inositol monophosphate was obtained from the California Foundation for Biochemical Research (Los Angeles, U.S.A.) and sodium phytate from L. Light and Co. Ltd. (Colnbrook, Buckinghamshire, England). Inositol diphosphate, inositol triphosphate and glycerylphosphorylinositol were prepared (Dawson & Dittmer, 1960) and phosphatidyl inositol (monophosphoinositide) was isolated from liver (Dawson, 1958). Bornesitol (1:0-methyl mesoinositol) was kindly given by Professor S. J. Angyal (Sydney).

RESULTS

Recovery of inositol from yeast

Table 1 shows the percentage distribution of the inositol recovered from *Kloeckera brevis* after its fractionation into water soluble, lipid and residual fractions. In two of the experiments the total recovery of inositol was measured and amounted to 95 and 99 % of that added to the growth medium; no detectable inositol remained in the medium. Although the percentage distribution of the inositol recovered showed appreciable variation in different cultures, it is clear that only a minor percentage of the combined inositol was extracted from whole yeast cells with trichloroacetic acid and lipid solvents, leaving the major portion in the insoluble residue.

Table 1. *Percentage distribution of inositol in fractions from* Kloeckera brevis

	Results as % of total inositol recovered					
	Whole yeast cells			Yeast disrupted twice in Hughes press		
Trichloroacetic acid extract	8	7	14	32	31	38
Chloroform + methanol extract	7	16	3	23	14	34
Acidified chloroform + methanol extract	20	20	4	20	51	15
Insoluble residue	65	57	79	25	4	13

The yeast cells proved to be particularly difficult to disrupt as judged by their microscopic appearance after Gram staining. Boiling with solvents or water, freezing and thawing or shaking in a Mickle shaker proved only partially effective. Treatment in a MSE Mullard Ultrasonic disintegrator with a 60 watt power output was ineffective, but partial breakage (80 %) was obtained after 50 min. in a Mullard instrument with a 500 watt output (the latter kindly performed by Dr R. Davies, Microbiological Unit, Department of Biochemistry, University of Cambridge). Crushing in a Hughes (1951) press proved to be reasonably effective and two treatments led to 70–90 % breakage of the yeast cells.

When yeast cells which had been partially disrupted on a Hughes press were fractionated the distribution picture of inositol changed considerably: the water-soluble fraction changed from 7–14 % of the total inositol to 31–38 %, and there was an appreciable decrease in the percentage of residual inositol (from 57–79 % to 4–25 %). In addition, the total lipid-inositol extracted with neutral and acidified chloroform + methanol showed some increase from 7–36 % to 43–65 %.

Composition of the water-soluble inositol fraction. This fraction was investigated by using ^{3}H-inositol labelled yeast which had been disrupted in a Hughes press. Chromatography and ionophoresis of aqueous extracts showed that the fraction was multiple, consisting of one major and two minor radioactive components. None of the components was sedimented on centrifuging at 78,000–173,000 *g* for 30 min. On ionophoresis at pH 3·6, the major ^{3}H component did not move, while both the minor components moved towards the anode, the fastest having a mobility identical with that of an internal inositol monophosphate marker (Fig. 2). This minor component contained phosphorus and its identification was confirmed by finding an exact coincidence of the radioactive peak with that of an added inositol monophosphate marker on paper chromatography in methanol + formic acid + water

(80 + 13 + 7; v/v) solvent, R_F 0·45. The amounts and proportions of the second minor spot varied from preparation to preparation. Its ionophoretic mobility at pH 3·6 was similar to that of glycerylphosphorylinositol but it could be readily separated from this compound on paper chromatography with an ethanol + NH_3 solvent.

The separated neutral major ^3H-component was distinguished from free inositol by paper chromatography in both the propanol + ethyl acetate + water and isopropanol + acetic acid + water solvents, when it ran in both systems more slowly than the free hexitol (Fig. 3). Very small amounts of free ^3H-inositol were occasionally seen in these chromatograms as well as the unknown component (Fig. 3). This

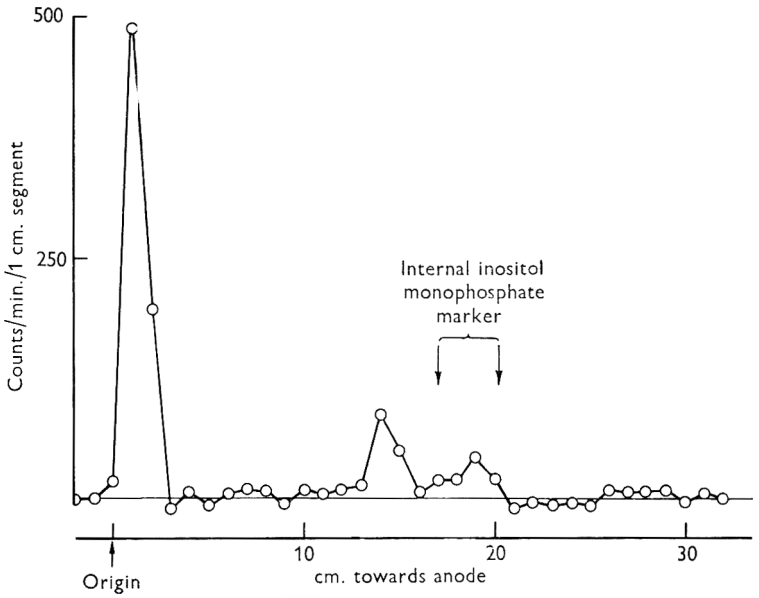

Fig. 2. Separation of water-soluble ^3H-labelled inositol derivatives from yeast. Ionophoresis at pH 3·6 for 100 min. at 50 volts/cm. The internal inositol monophosphate marker was revealed by spraying to detect phosphorus.

latter compound was not capable of supporting the growth of *Kloeckera brevis* when added to a growth medium under standard assay conditions. However, it did so after a fairly vigorous acid hydrolysis, e.g. 6 hr. in 2 N-HCl at 105°. This suggests that free mesoinositol was liberated, since the growth of the organism was specifically dependent on the presence of this substance in the growth medium. This was confirmed by acid hydrolysis of the compound, followed by paper chromatography of the hydrolysate in propanol + ethyl acetate + water. A ^3H-labelled compound was formed which reacted with alkaline silver nitrate (Trevelyan *et al.* 1950) and whose radioactivity migrated exactly to the same position as that of an internal inositol marker. There was no evidence of the formation on acid hydrolysis of other alkaline silver-reducing polyols, such as glucose or mannose.

The unknown component was readily separated on ionophoresis from the following phosphorylated derivatives: inositol monophosphate, inositol diphosphate, inositol triphosphate, glycerylphosphorylinositol, phytic acid. No phosphate was

detected on spraying chromatograms nor were ninhydrin-reacting groups apparent. This is consistent with its failure to move on ionophoresis, and its ability to pass through a monobed resin (Amberlite MB 1). The compound was chromatographically distinguished from another series of naturally occurring inositol derivatives, the monomethylethers. Thus, in isopropanol + acetic acid + water and in propanol + ethyl acetate + water, it ran more slowly than free inositol and much more slowly than bornesitol (1:0-methyl mesoinositol; Fig. 3). In general all the monomethyl ethers of inositol appear to run faster than free inositol in solvents of this type (Posternak, Reymond & Haerdi, 1955; Angyal, McHugh & Gilham, 1957).

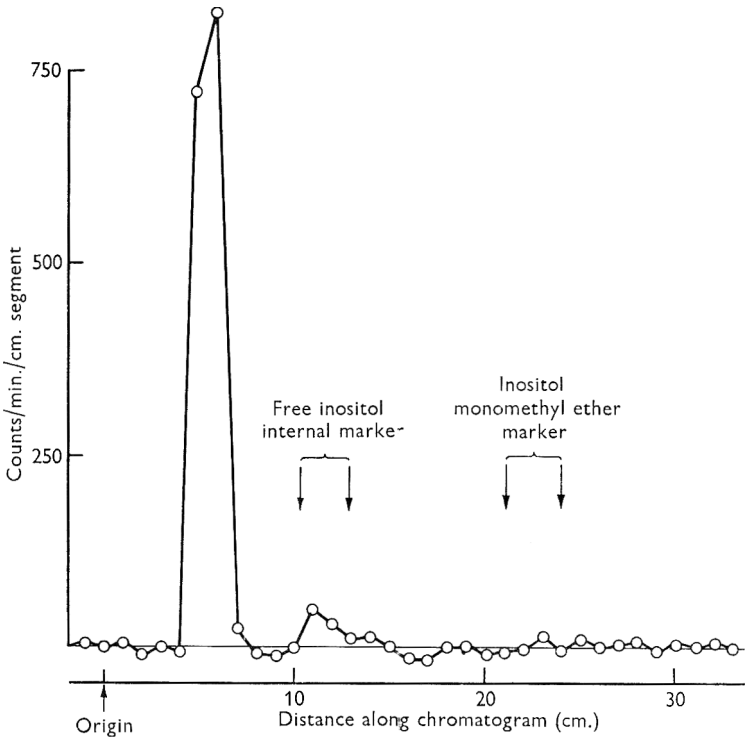

Fig. 3. Separation of major water-soluble ^{3}H-labelled inositol derivative from free inositol and inositol monomethyl ether. Solvent *n*-propanol + ethyl acetate + H_2O; 48 hr. descending paper chromatography. Markers were detected by spraying to reveal polyols.

Examination of the lipid fractions. When the lipids extracted both with neutral solvents and acidified solvents from ^{32}P-labelled yeast were hydrolysed for 10 min. with 5N-HCl at 100°, ionophoresis of the hydrolysis products at pH 3·6 separated a small radioactive spot with a mobility identical with that of inositol monophosphate. A large amount of glycerophosphate was also produced. After de-acylation by mild alkaline hydrolysis followed by chromatography and ionophoresis (Dawson, 1960), the material extracted with neutral lipid solvents gave spots on radioautography which were in the typical position of glycerylphosphorylinositol and inositol monophosphate. These are alkaline degradation products of phosphatidyl inositol (Dawson, 1960). The same general picture was observed after labelling the

yeast with ^{3}H-inositol. However, after alkaline treatment of the ^{32}P-labelled material extracted with acidified lipid solvents no glycerylphosphorylinositol or phosphoryl inositol was detected on chromatography and ionophoresis, followed by radioautography. When an acidified chloroform + methanol solution of the lipid from ^{3}H-labelled yeast was shaken with 0·2 vol. of water, the ^{3}H became concentrated at the interface on centrifugation. This is the behaviour to be expected if the ^{3}H-inositol were present as in a lipoprotein (Lebaron & Folch, 1956). In the neutral lipid extract, large amounts of lecithin and phosphatidyl ethanolamine were detected with smaller amounts of phosphatidyl serine and a polyglycerolphospholipid of a cardiolipid type. These were not present in the material extracted with acidified solvents.

Since mannose-containing phosphoinositides have been described in bacteria (Vilkas & Lederer, 1956; Nojima, 1959), samples of the two lipid fractions were hydrolysed for 48 hr. in 5N-HCl at 105° and the hydrolysate tested chromatographically for mannose. Neither mannose nor glucose was detected on spraying for polyols, although inositol and glycerol were clearly revealed.

DISCUSSION

The present results show that *Kloeckera brevis* only yielded a large part of its combined inositol to extracting solvents when it has been previously disrupted by mechanical shearing in a Hughes press. This mechanical shearing was associated with a microscopically visible disruption of the yeast cell and a disappearance of Gram-positive staining. Many other treatments of the yeast did not give satisfactory disruption and the barrier to effective inositol extraction remained. As the two treatments in the Hughes press only gave a degree of breakage which was visually estimated at between 70 and 90%, it would seem reasonable to suppose that completely disrupted cells would yield practically all their combined inositol to trichloroacetic acid and lipid solvents.

Of the water-soluble inositol extracted from the yeast, a major portion consisted of a neutral derivative of inositol which yielded mesoinositol on acid hydrolysis. This could not be equated with any known naturally-occurring water-soluble form of combined inositol, e.g. phytic acid. The yeast cannot use this water-soluble combined inositol to support growth, which suggests either a permeability barrier or the lack of a suitable enzyme for converting the compound to free mesoinositol.

It is possible that the minor component in the water-soluble fraction, inositol monophosphate, is a breakdown product of the phosphatidyl inositol present in the yeast. In mammalian tissues inositol monophosphate is not on the biosynthetic pathway of phosphatidyl inositol (Agranoff, Bradley & Brady, 1958; Paulas & Kennedy, 1960); but recently enzymes have been described which produce it by hydrolysis of this lipid (Dawson, 1959; Kemp, Hübscher & Hawthorne, 1959).

Although the de-acylation products of the inositide present in the yeast were chromatographically identical with those of phosphatidyl inositol, a positive identification must await the isolation of the lipid itself. Phosphatidyl inositol has been isolated from the non-inositol-dependent yeast *Saccharomyces cerevisiae* (Hanahan & Olley, 1958) and phosphatidyl-inositomannosides from *Mycobacterium tuberculosis* (Vilkas & Lederer, 1956; see also Nojima, 1959). The latter compounds,

however, would yield mannose on acid hydrolysis and this sugar was absent from the present lipid extracts.

The growth of *Kloeckera brevis* presumably ceases in inositol deficiency because the cells are not able to synthesize one or other of the inositol-containing components which are essential for budding and development of daughter cells. From the properties of these components and their tight attachment to the yeast cell, it seems more probable that they are essential structural elements rather than coenzymes in some metabolic process essential to yeast growth. This is supported by studies with the inositol-dependent yeast *Saccharomyces carlsbergensis* where inositol deficiency caused the formation of abnormal cell walls with the failure of daughter cells to separate, leading to large cellular aggregates (Smith, 1951; Ghosh, Charalampous, Sison & Borer, 1960). At the same time no change in the intermediary metabolism could be detected.

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The Preservation of Leptospires by Drying from the Liquid State

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SUMMARY

Four strains of Leptospira were preserved by drying, *in vacuo*, small volumes (0·04 ml.) of suspension of the organism from the liquid state. The leptospires were suspended and dried in a mixture of equal parts of Korthof's medium and 20% (w/v) glucose. Recoveries of the organisms after storage for one year were appreciably higher from desiccates stored at 4° than from those stored at about 20°.

INTRODUCTION

A wide range of micro-organisms is now routinely preserved in the dried state. This is not so however for leptospires, which are maintained by animal passage, in culture, or in frozen suspensions. The recovery of leptospires from desiccates after storage for 2 years has been reported previously (Annear, 1958*a*), but the results revealed the method to be of doubtful value for long-term preservation of the organisms. Moreover, the drying method was a relatively complex one. The present paper describes the drying of leptospires by a simpler method which the results indicate to be more efficient. Briefly the procedure consists of drying *in vacuo* and over P_2O_5 small volumes of microbial suspension in ampoules which are immersed in a water bath at room temperature to prevent freezing during evaporation. The method has been previously applied to the preservation of other bacteria (Annear, 1958*b*) and the protozoan *Strigomonas oncopelti* (Annear, 1961).

METHODS

The four strains of Leptospira investigated are shown in Table 1. The organisms were grown in 200 ml. volumes of Korthof's medium at 28°, and 3–6 days after the first appearance of turbidity were harvested by centrifuging the culture in 1 oz. bottles. The deposited organisms were resuspended and pooled in about 2 ml. of the supernatant fluid, and with a syringe-pipette (Annear, 1956) single drops were delivered into ampoules (Edwards High Vacuum Ltd., E30/C17) which had been previously charged with one drop of 20% (w/v) glucose solution; the drop volume in each case was about 0·02 ml. Cotton-wool plugs were pushed down the necks of the ampoules, which were then connected to a manifold containing P_2O_5 (Annear, 1958*b*). The bottoms of the ampoules were immersed in a water bath at room temperature (about 20°) and the suspensions dried from the liquid state. After pumping overnight, the ampoules were constricted, placed on a vertical manifold

and sealed *in vacuo* after a further 6 hr. of pumping. The sealed ampoules were stored at 4° or at room temperature (usually about 20°, but within a range of 17–30°).

Rehydration of the desiccates was effected by resuspending the dried organisms in about 0·5 ml. of Korthof's medium in the ampoule and then pipetting this volume into a further 9·5 ml. volume of the same medium. A drop of the smaller volume was examined microscopically. The 10 ml. volume of suspension was divided into four samples which were incubated at 31°. Daily examinations of the tubes were made and those showing turbidity were examined microscopically.

Table 1. *Recoveries of leptospires after drying and storage for* 12 *months*

Organism	Expt.	Storage temperature	No. of desiccates examined	No. of days* for cultures to show visible growth
L. icterohaemorrhagiae (Jackson)	1*a*	4°	1	6
	b	Room	1	8
	2*a*	4°	1	6
	b	Room	1	8
	3	4°	3	5, 5, 5
	4*a*	4°	2	5, 5
	b	Room	2	7, 8
	5*a*	4°	1	8
	b	Room	3	8, 10, 11
L. icterohaemorrhagiae (W)	6	4°	1	6
L. canicola (Berlin)	7	4°	1	6
L. pomona (Staines)	8	4°	1	9
	9	4°	2	7, 7

* The values in the column are means of the sets of four samples.

RESULTS

Numerous desiccates have been examined during 12 months of storage, all of them yielding living leptospires. The results shown in Table 1 are those from ampoules examined at the end of this period. Microscopically all of the rehydrated desiccates showed large numbers of inert leptospires. However, actively motile leptospires were seen on rehydrating many of the desiccates which had been stored at 4° and in some of those stored at room temperature. No attempt was made to assess the fraction of active leptospires but in some of the preparations there were hundreds of them in each low-power field (× 200). When first rehydrated the motile organisms did not have the morphology of actively growing leptospires but appeared somewhat distorted and compressed. Within a few hours, however, many normal forms were visible. On culture, all desiccates, including those in which no active forms were seen, yielded growth. An occasional tube among the sets of four samples became contaminated. Except for Expt. 5*b* (Table 1), there was no more than a difference of one day among the recovery periods of each set of samples or between the means of sets of samples from replicate desiccates. In Expt. 5*b* there were differences of as many as three days in both cases. As in the previous study (Annear, 1958*a*)

there were considerable differences between recovery periods from ampoules stored at room temperature and those stored at 4°, and these differences will undoubtedly increase with further storage.

DISCUSSION

The results from storage at 4° are encouraging and suggest that the method of drying is worth further study for long-term preservation of leptospires. The recovery periods shown in Table 1 are little or no longer than those from desiccates rehydrated at the beginning of the storage period, indicating that there has been no appreciable deterioration during storage. The conditions of drying so far have been quite arbitrary and the critical investigation of such factors as the age of the cultures and the composition of the suspending medium may well lead to more efficient drying of the organisms and to higher storage survival, particularly at room temperature.

In previous work (Annear, 1958*b*) considerable variation occurred among recoveries from replicate desiccates of salmonellas dried from the liquid state on glass surfaces of ampoules, whereas the results were more uniform when the same suspensions were dried on tufts of cotton wool. It should be profitable to make such comparisons with leptospires. Also the concentration of organisms in the suspension to be dried might be increased by decreasing the volume of the supernatant fluid in which the organisms are suspended after centrifugation, and this might result in more rapid recoveries after drying. Similar results to those shown here were obtained when glucose was added to the suspension of the organism before it was delivered into the ampoules. Such a procedure is simpler, in that it involves only a single charging of the ampoules, but the technique as described here does constitute a convenient method for testing a range of preserving media in the one experiment.

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The Osmotic Requirements for Growth of Mycoplasma

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SUMMARY

Alteration of the tonicity of a modified Edward medium by means of NaCl or other solutes resulted in very marked effects on the growth of several Mycoplasma strains. With both fluid and solid media, all grew best at about 10 atmospheres osmotic pressure (water activity, a_w, 0·9925). The most exacting species, *Mycoplasma gallisepticum*, failed to grow outside the range 6·8–14 atmospheres (a_w 0·995–0·990), while the least exacting, *M. laidlawii*, multiplied at up to 27 atmospheres (a_w 0·980). Osmotic requirements were not appreciably altered by serial growth in hypertonic or hypotonic media but were, to some extent, conditioned by the serum content of the medium.

INTRODUCTION

The plasticity and pleomorphism of organisms of the Mycoplasma group are usually attributed to a lack of a rigid cell wall (Klieneberger & Smiles, 1942; Klieneberger-Nobel, 1954). This concept is supported by electron micrographic studies (Smith, Hillier & Mudd, 1948; Klieneberger-Nobel & Cuckow, 1955) and by evidence that these organisms are deficient in the 'mucocomplex' found in bacterial cell walls (Kandler & Zehender, 1957; Plackett, 1959). It is generally stated that as a consequence of this deficiency the Mycoplasma are very susceptible to osmotic shock (Freundt, 1952; Chu, Horne & Cosslett, 1956), although this view has been questioned (Smith & Sasaki, 1958; Butler & Knight, 1960*a*; Adler & Shifrine, 1960). Very little attention, however, has been paid to the osmotic requirements for growth of Mycoplasma in artificial culture. Rodwell (1956) noted that the tonicity of the medium was critical for *Mycoplasma mycoides* var. *mycoides* and Adler & Shifrine (1960) referred to unpublished observations on the osmotic requirements of avian species. This paper describes investigations of the osmotic requirements for growth of several species of Mycoplasma.

METHODS

Strains. *Mycoplasma mycoides* var. *mycoides* (P.G.1), *M. mycoides* var. *capri* (P.G.3), *M. laidlawii*, strain A (P.G.8), *M. bovigenitalium* (P.G.12), *M. gallinarum* (P.G.16) and *M. gallisepticum* (P.G.31) were obtained from the collection maintained in these Laboratories (Edward & Freundt, 1956). An egg-adapted (T3) strain of *M. mycoides* var. *mycoides* was obtained through the courtesy of Dr S. E. Piercy and Mr G. White. The M.R. strain of an unnamed Mycoplasma species from the respiratory tract of a pig was kindly provided by Dr P. Whittlestone (Cambridge

Veterinary School). A strain of *M. bovigenitalium*, associated with bovine mastitis, was kindly provided by Mr I. Davidson of the Central Veterinary Laboratory, Weybridge.

Media. Edward medium (Butler & Knight, 1960*b*) was used for some growth experiments and in the solid form, with 1·2 % (w/v) agar (New Zealand, Difco), for viable counts. A basal medium of low tonicity ('dilute Edward medium'), prepared by omission of added NaCl from Edward medium and dilution of the final medium, is described under Results.

Estimation of tonicity. Tonicity was estimated by means of freezing-point depression measurements made with a Beckman-type thermometer. The water vapour values for frozen and for liquid water at a given temperature were obtained from standard tables (*Handbook of Chemistry and Physics*, 1958). The water activity (a_w; see Scott, 1953, 1956) of any medium was then calculated as the ratio of these two vapour pressure values at the freezing temperature of the medium; a_w was converted to osmotic pressure and then adjusted to the theoretical value for 25° by means of the formulae given by Scott (1956).

The tonicity of a medium of known a_w was raised to required values by addition of either NaCl, KCl, Na_2SO_4, sucrose, or a balanced salts solution (NaCl: KCl: Na_2SO_4: $MgCl_2$ in molal ratios 5:3:1:1) by using the tables of Robinson & Stokes (1955) as described by Scott (1953, 1956). Table 1 shows the actual amounts of solutes used to enhance the tonicity of the dilute Edward medium.

Colony counts. These were performed by the method of Butler & Knight (1960*a*). Where samples of Mycoplasma growing in media of varied tonicity were being tested, the dilutions of such samples for viable counts were normally made in dilute Edward medium with osmotic pressure adjusted to 10 atmospheres.

Opacity of suspensions. The opacity of Mycoplasma culture suspensions was measured with a photoelectric nephelometer head and galvanometer (Evans Electroselenium, Ltd., Essex; EEL), using standardized 6 in. × $\frac{5}{8}$ in. test tubes and reading against the EEL Perspex standard opaque tube, with blanks of uninoculated medium. For each strain, growth in different media was compared on the basis of maximum degrees of opacity and of arbitrary growth rates based on the rate of change of opacity (nephelometer units/hr.) over the whole of the active growth period. Two strains, *Mycoplasma bovigenitalium* and *M. gallinarum*, produced in fluid culture an insoluble refractile material characteristic of those Mycoplasma which are able to form 'film and spots' in solid medium (Edward, 1950). This was not considered to affect the validity of the nephelometric tests for the present purposes.

Growth experiments. Inocula were prepared from surface growth of Mycoplasma on Edward agar medium suspended in dilute Edward medium. For growth curves measured by viable counts, 10 ml. lots of test media contained in 1 oz. screw-capped bottles were seeded with 0·1 ml. of suspensions adjusted to give initial viable counts of about 10^5 viable units/ml. After inoculation, cultures were incubated at 37° in air, and at intervals 0·1 ml. samples were taken for viable counts. For experiments in which growth was measured by nephelometry, approximately 10 times larger inocula were used and the inoculated cultures were contained in EEL 6 in. × $\frac{5}{8}$ in. calibrated tubes with aluminium caps. These were incubated in a 37° water bath and removed at intervals for opacity readings in the nephelometer.

RESULTS

Freezing-point measurements showed that Edward medium had an osmotic pressure of about 10 atmospheres. The ox heart infusion broth on which this medium is based contains 0·5 % (w/v) of added NaCl (Butler & Knight, 1960*b*) and when this NaCl was omitted the full medium was approximately isotonic with physiological fluids (6·8 atmospheres). A 40 % (v/v) dilution in distilled water of this salt-deficient medium, having an osmotic pressure of 2·7 atmospheres, was used as a standard medium and is referred to throughout this paper as 'dilute Edward

Table 1. *Amounts of various solutes added to dilute Edward medium to give required values of tonicity*

	Tonicity expressed as		Concentration of solutes (g./100 ml. medium)				
Medium	Water activity (a_w) at 25°	Osmotic pressure in atmospheres (calculated from a_w)	NaCl	KCl	Na_2SO_4	Balanced salts NaCl:KCl:Na_2SO_4:$MgCl_2$	Sucrose
1	0·998	2·7	0	0	0	0	0
2	0·997	4·1	0·17	0·22	0·33	0·084:0·064:0·041:0·027	1·9
3	0·995	6·8	0·52	0·67	0·99	0·25 :0·19 :0·12 :0·082	5·6
4	0·9925	10·2	0·97	—	—	—	—
5	0·990	13·6	1·4	1·8	2·8	0·67 :0·52 :0·33 :0·22	15
6	0·980	27	3·20	4·2	7·0	1·5 :1·6 :0·73 :0·49	32
7	0·970	41	4·95	—	—	—	—
8	0·960	55	6·7	8·9	16	3·2 :2·4 :1·5 :1·0	63

Table 2. *Effect of tonicity of fluid medium on growth of Mycoplasma*

		Tonicity as osmotic pressure (atmospheres)						
Strain of *Mycoplasma*	Type of measurement	2·7	4·1	6·8	10	14	27	41
M. laidlawii	Growth rate*	0·5	1·0	1·2	1·2	1·6	0·3	0
	Maximum opacity†	13	21	27	36	35	11	—
M. mycoides var. *mycoides*, Strain PG1	Growth rate	0	0·2	0·5	0·7	0·7	0	0
	Maximum opacity	0	9	15	25	24	0	0
M. mycoides var. *mycoides*, Strain T/3	Growth rate	0	0	0·2	0·7	0·7	0	0
	Maximum opacity	0	0	⩾ 7	12	11	—	—
M. mycoides var. *capri*	Growth rate	0·1	0·4	0·5	0·7	0·6	0	0
	Maximum opacity	13	19	27	29	25	0	0
M. bovigenitalium	Growth rate	0	0·4	0·4	0·6	0·5	0	0
	Maximum opacity	0	9	22	32	24	0	0
M. gallisepticum	Growth rate	0	0	0·8	1·1	1·1	0	0
	Maximum opacity	0	0	8	17	17	0	0
M. gallinarum	Growth rate	0·5	1·3	1·3	1·3	0·7	0	0
	Maximum opacity	41	37	38	42	38	0	0
Porcine Mycoplasma, Strain MR	Growth rate	0	0·3	0·3	0·5	0·5	0	0
	Maximum opacity	0	10·5	11·5	17·5	17	0	0

* Approximate rate of change (nephelometer units/hr.) during period of active increase in opacity.
† Nephelometer units.

medium'. To this, NaCl or other solutes were added to raise the tonicity to required values (Table 1). Solid medium was prepared from this medium by addition of 1·2% (w/v) agar (New Zealand, Difco).

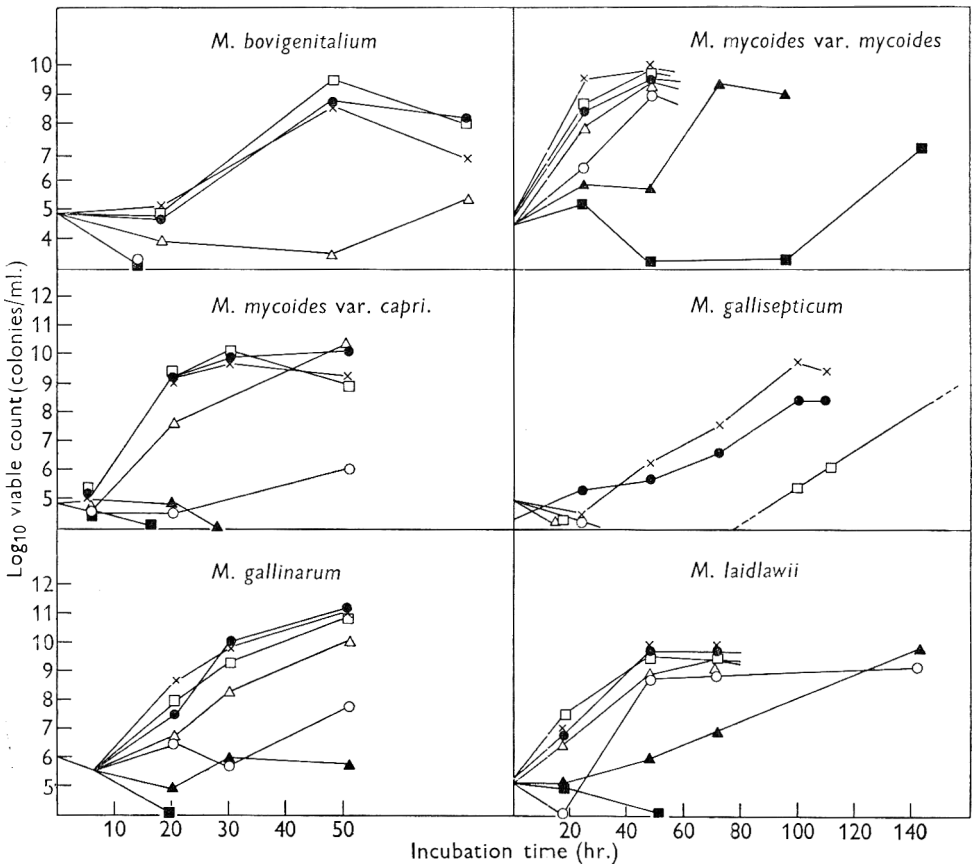

Fig. 1. Effect of tonicity of medium on growth of Mycoplasma. Growth of several species of Mycoplasma in dilute Edward medium after adjustment of tonicity with NaCl to 2·7 (○), 4·1 (△), 6·8 (□), 10 (×), 14 (●), 27 (▲), or 41 (■) atmospheres osmotic pressure.

Growth in fluid medium

A number of Mycoplasma strains which showed sufficient growth from large inocula to be measured by nephelometry were tested in media of different osmotic strengths. Table 2 summarizes the results obtained from these experiments. The relation of growth rate and final opacity to the osmotic strength of the medium was very marked for all strains. This was supported by other experiments in which growth of smaller inocula was followed by viable counts (Fig. 1). With the exception of a laboratory strain of *Mycoplasma mycoides* var. *mycoides*, which gave rather variable results, the results obtained by nephelometry and by viable counts were strikingly similar. The optimal osmotic pressure was always between 6·8 and 14 atmospheres, usually about 10 atmospheres, which is slightly hypertonic in the physiological sense. Sensitivity to osmotic conditions differed greatly between various

strains. *M. gallisepticum* did not grow outside the narrow range of 6·8–14 atmospheres, whereas for the saprophytic *M. laidlawii* the limits were ≤ 2·7–27 atmospheres. Except for the laboratory strain of *M. mycoides* var. *mycoides*, only *M. laidlawii* showed multiplication, within the experimental period, at more than 14 atmospheres. Growth of *M. mycoides* var. *mycoides* was greatly retarded at 41 atmospheres, but in several other experiments no growth of this strain occurred at this value of tonicity (e.g. Table 5). For most species slightly suboptimal conditions resulted only in slower growth rates but greater extremes of tonicity sometimes prolonged the lag phase of growth (Fig. 1).

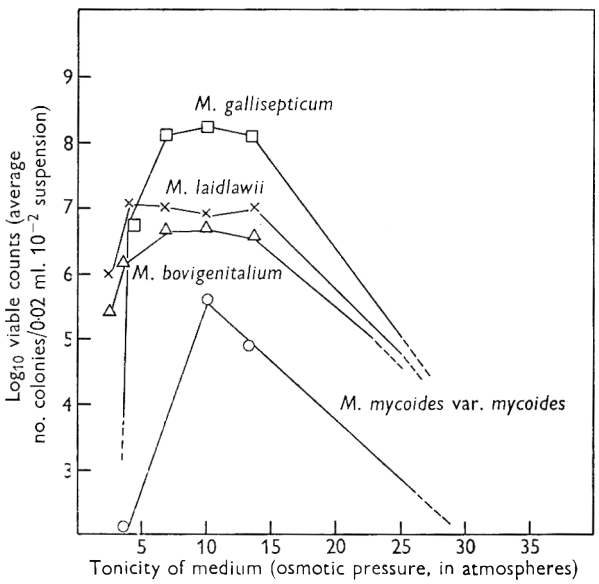

Fig. 2. Effect of tonicity of agar medium on colony counts of Mycoplasma suspensions.

Growth on solid media

Growth on solid media was estimated by the number of colonies produced from a given dilution of Mycoplasma suspension in 0·01 M-phosphate-buffered (pH 7·4) physiological saline. Preliminary tests showed that the tonicity of the fluid in which the inoculum was diluted had virtually no effect on the results. As with fluid medium, optimal growth of colonies was provided by an osmotic pressure of about 10 atmospheres (Fig. 2). Except for *Mycoplasma mycoides* var. *mycoides*, which was more sensitive to the tonicity on solid medium, the limiting osmotic conditions were similar to those found for growth in fluid medium.

The requirement for an optimal osmotic pressure of 10 atmospheres was confirmed under conditions of primary isolation when samples of milk from a cow suffering from a mastitis due to *Mycoplasma bovigenitalium* (Davidson & Stuart, 1960) were plated at various dilutions on solid media of different tonicities (Table 3). At high inoculum dilutions this organism was even more exacting osmotically than the laboratory strain of *M. bovigenitalium* (Figs. 1, 2).

Adjustment of tonicity with solutes other than NaCl

To investigate the possibility that NaCl was required specifically, other solutes including Na_2SO_4, KCl, sucrose and a balanced salts solution (see Methods), were used to increase the tonicity of dilute (solid) Edward medium (Table 1). The results of tests with three Mycoplasma strains are shown in Table 4. In general these showed the same relationship between the tonicity of the medium and colony counts as had been obtained in experiments with NaCl (Fig. 2). Growth was always maximal between 6·8 and 14 atmospheres osmotic pressure.

Table 3. *Effect of tonicity of agar medium on primary isolation of* Mycoplasma bovigenitalium *from milk*

Osmotic pressure of medium (atmospheres)	Average number of colonies per 0·02 ml. inoculum dilutions after incubation at 37° for 10 days	
	10^{-3}	10^{-4}
2·7	0	0
4·1	1·3	0
6·8	1000	10
10	1000	150
14	1000	110
27	0	0

Table 4. *Adjustment of tonicity of agar medium with solutes other than NaCl*

Scoring shows growth produced by 10^{-2} dilution of cultures of three Mycoplasma species.

Scoring: + About 10 colonies per 0·02 ml. inoculum. ++ About 100–1000 colonies per 0·02 ml. inoculum. +++ About or greater than 10,000 colonies per 0·02 ml. inoculum.

Mycoplasma species	Solutes	Osmotic pressure of medium (atmospheres)					
		2·7	4·1	6·8	14	27	55
M. laidlawii	Na_2SO_4	+++	+++	+++	+++	0	0
	KCl	+++	+++	+++	+++	+++	0
	Balanced salts mixture*	+++	+++	+++	+++	0	0
	Sucrose	0	++	++	++	0	0
M. mycoides var. *mycoides*	Na_2SO_4	0	+++	+++	+++	0	0
	KCl	0	0	0	0	0	0
	Balanced salts mixture*	0	0	+++	+++	0	0
	Sucrose	0	0	+++	+++	0	0
M. gallisepticum	Na_2SO_4	++	++	+++	+++	0	0
	KCl	+++	+++	+++	+++	+++	0
	Balanced salts mixture	+++	+++	+++	+++	0	0
	Sucrose	+	+	++	++	0	0

* NaCl:KCl:Na_2SO_4:$MgCl_2$ in molal ratios 5:3:1:1 (Methods).

Adaptation of osmotic requirements

Attempts were made to alter the osmotic requirements of the laboratory strain of *Mycoplasma mycoides* var. *mycoides* by continued subculture at different values of medium tonicity. From an experiment in which this strain showed multiplication at 27 atmospheres and at 2·7 atmospheres in fluid medium, sublines were made and maintained for 12 twice-weekly subcultures at each of these respective values of tonicity, and also at optimal tonicity (10 atmospheres). The osmotic requirements, when tested in fluid medium at the end of this period, were very similar to those of the 'unadapted' strain (Table 5), except for a slightly diminished tolerance of hypertonic conditions shown by the organisms maintained at low tonicity and a slightly greater tolerance of hypertonicity by organisms maintained at high tonicity.

Table 5. *Effect of repeated subculture at different values of tonicity on osmotic requirements of* Mycoplasma mycoides *var.* mycoides

Increase in viable counts (log. colonies/ml.) after 19 hr. incubation in fluid media of different tonicities. Standard inocula prepared from subcultures of *M. mycoides* var. *mycoides* maintained for twelve passages at 2·7, 10 or 27 atmospheres.

Culture maintained at (atmos.):	Osmotic pressure of test medium (atmospheres)						
	2·7	4·1	6·8	10	14	27	41
	log. colonies/ml.						
2·7	0·6	2·2	2·7	1·5	0* delayed growth	Decrease	Decrease
10	0	1·4	2·0	2·0	1·8	Decrease	Decrease
27†	2·2	3·2	4·1	3·8	3·1	0·5	Decrease

* Still in log phase at 19 hr.

† A smaller inoculum was used; hence the increases at 19 hr. were greater than for the other two inocula.

Effect of serum content of medium on osmotic requirements

Mycoplasma laidlawii was able to grow in dilute Edward medium without serum, and its osmotic requirements remained the same under these conditions. Other species, however, failed to grow in the complete absence of serum, irrespective of the tonicity of the medium. The effect of decreased serum concentrations was examined for one such species, *M. bovigenitalium*, which in dilute Edward medium (containing 8 %, v/v, serum) was able to grow only at osmotic pressures of between 4·1 and 14 atmospheres (Fig. 1; Table 2). Equivalent suspensions of this organism were inoculated into dilute Edward medium in which the serum content was varied between 0·25 and 16 % (v/v). Osmotic pressure was adjusted to 4·1, 6·8 and 10 atmospheres, growth at 14 atmospheres having been found in a preliminary experiment to be almost identical with that at 10 atmospheres for all serum concentrations.

Growth curves for each medium indicated an interrelationship between the serum content of the medium, its tonicity, and the growth rate of *Mycoplasma bovigenitalium* (Fig. 3). At the highest serum concentrations the rates at 6·8 and 10 atmospheres were similar, while growth at 4·1 atmospheres was moderate but sub-optimal. With

decreasing serum concentration the differences in growth at the three tonicity values became more marked. Thus in 1 % (v/v) serum medium growth at 10 atmospheres was still nearly optimal; it was appreciably slower at 6·8 atmospheres and at 4·1 atmospheres had decreased to zero. At 0·25 % of serum, growth at 10 atmospheres had diminished markedly while that at 6·3 atmospheres was almost at zero value.

Effect of spermine

The polyamine spermine, which protects certain bacteria against adverse osmotic conditions (Mager, 1959), had no effect on the osmotic requirements of three Mycoplasma species growing in fluid media. *Mycoplasma gallisepticum* was tested with 2 μM, *M. gallinarum* with 0·4 μM, and *M. bovigenitalium* at 0·2–20 μM spermine phosphate.

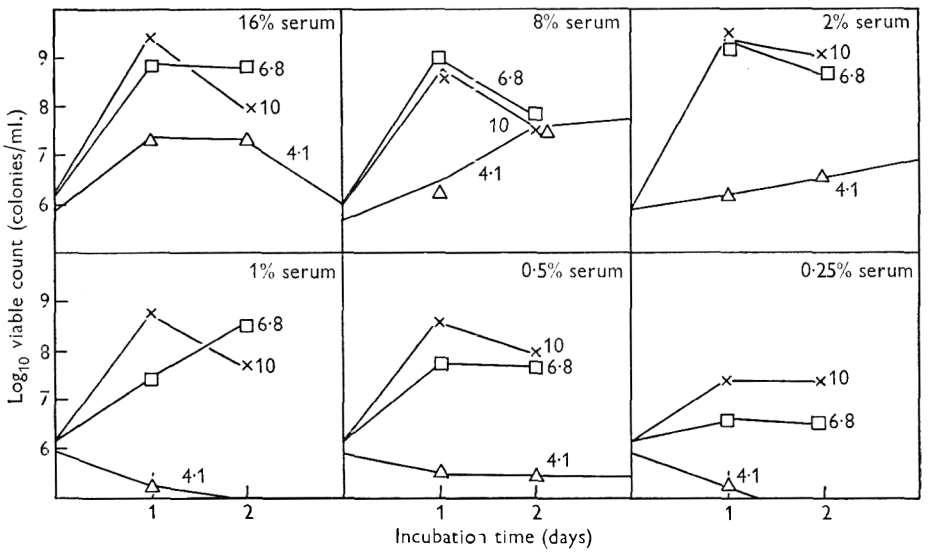

Fig. 3. Effect of serum concentration on growth of *Mycoplasma bovigenitalium* in fluid medium of different tonicities. Numbers adjacent to each growth curve refer to tonicity of medium expressed as osmotic pressure (atmospheres).

DISCUSSION

The above experiments with several species of Mycoplasma confirm the suggestion of Adler & Shifrine (1960), based on their own observations with avian Mycoplasma and on those of Rodwell (1956) with *Mycoplasma mycoides* var. *mycoides*, that this group can multiply in artificial media only within very restricted values of tonicity and has an optimal osmotic pressure requirement of about 10 atmospheres. The upper limit for growth appears to be about 27 atmospheres for *M. laidlawii* and usually 14 atmospheres for non-saprophytic strains. The lower limits are wider, several strains being able to grow sub-optimally at about 2·7 atmospheres, with sensitive strains such as *M. gallisepticum* and *M. maculosum* unable to multiply at less than 6·8 atmospheres. When expressed as water activity (Table 1) these limits (upper 0·990–0·980 a_w; lower 0·998–0·995 a_w) are seen to be much narrower than those for a wide range of bacteria as compiled by Scott (1956). Even a very delicate bacterial species such as *Vibrio metchnikovi* is capable of multiplication between

0·970 and 0·999 a_w (Marshall & Scott, 1958). The fact that Mycoplasma strains are more exacting in osmotic requirements than bacteria is consistent with their lack of a rigid cell wall. It is of interest that initial viable counts made 5–15 min. after inoculation of fluid media of varying tonicity were very similar (Fig. 1), suggesting that these organisms suffered little osmotic shock in any of these media. This is in accord with suggestions that the group is less susceptible to osmotic shock than is generally supposed (Smith & Sasaki, 1958; Butler & Knight, 1960*a*; Adler & Shifrine, 1960). Plackett (1959) pointed out that the disruptive forces due to a hypotonic environment could be relatively slight for organisms as small as Mycoplasma. Such forces should be expected to be more critical during growth and division of cells and it is, therefore, not surprising that the tonicity of the medium should be much more important for actual multiplication. It is not clear, however, whether the slower rates of multiplication at sub-optimal tonicities in the present experiments were due to death of a proportion of cells during or after division, or to impaired metabolism of all the cells in a culture under these conditions.

It may be significant that *Mycoplasma laidlawii*, which is able to grow without serum, had the least exacting osmotic requirements of the strains tested (Fig. 1). This is consistent with the fact that for one of the more exacting species, *M. bovigenitalium*, there was an interrelationship between serum concentration and tonicity requirements. High serum concentrations were necessary for growth in medium of low tonicity and hypertonic conditions for optimal growth at lower serum concentrations (Fig. 3). This relationship suggests that at least a partial role of serum is that of protection against adverse osmotic conditions. Rodwell (1956) showed that serum components, including cholesterol, protect suspensions of *M. mycoides* var. *mycoides* against lysis by surface-active substances. Further work on the osmotic effect of various components of serum, including cholesterol, which is a specific growth factor for Mycoplasma (Edward, 1954), may help to elucidate their role in the nutrition of this group of organisms.

From the practical point of view it is interesting that a newly isolated strain of *Mycoplasma bovigenitalium* showed much the same osmotic requirements as the laboratory lines of Mycoplasma (Table 3). This observation, together with the failure of subcultivation at extremes of tonicity to change the optimal osmotic requirements of *M. mycoides* var. *mycoides*, suggests that the special osmotic requirements of our laboratory strains of Mycoplasma are not simply a result of adaptation to the slightly hypertonic Edward medium.

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