J. gen. Microbiol. (1964), **36**, 333–339
Printed in Great Britain

Studies of Methionine Synthesis in *Coprinus lagopus*

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(*Received* 3 *March* 1964)

SUMMARY

When methionine was present in the growth medium of wild-type *Coprinus lagopus* the incorporation of isotope from ^{35}S-sulphate into both cysteine and methionine was greatly decreased. Under these conditions much of the cysteine formed must have arisen from methionine and it is concluded that the diminution of cysteine synthesis from sulphate is caused by the cysteine formed from the methionine and not directly by methionine itself.

Wild-type Coprinus accumulated large amounts of cystathionine when grown with methionine and this appeared to have arisen from the added methionine as isotope from ^{35}S-sulphate was not incorporated into the accumulated compound. The cysteine formed from methionine may have been formed from this accumulated cystathionine.

INTRODUCTION

In previous work from this laboratory (Lewis, 1961) the genetic system concerned with methionine synthesis in *Coprinus lagopus* was studied and in particular seven genes of the methionine synthesis pathway (as well as a number of suppressor genes) were mapped. From the nutritional characteristics of the mutants examined it appeared likely that methionine synthesis, as in other organisms (Davis, 1955), occurred via the formation of cysteine, cystathionine and homocysteine.

With other organisms, e.g. *Escherichia coli* (Roberts *et al.* 1955; Wijesundera & Woods, 1960), *Neurospora crassa* (Roberts *et al.* 1955) and *Salmonella typhimurium* (R. J. Rowbury, unpublished observations), the formation of methionine is controlled by methionine itself. Roberts *et al.* investigated the regulation of methionine synthesis in *E. coli* and *N. crassa* by measuring the incorporation of isotope from ^{35}S-sulphate into sulphur amino acids in the presence and absence of methionine. Methionine, in the growth medium, abolished incorporation of ^{35}S into the methionine of the proteins. In the present work, the effect of methionine on sulphate incorporation in Coprinus has been tested, to ascertain whether a similar effect occurs.

Auxotrophic mutants of micro-organisms frequently accumulate precursors of the required growth factors and such accumulations occur with certain methionine-requiring organisms. Thus one mutant strain of *Neurospora crassa* accumulated cystathionine in the mycelium (Horowitz, 1947) and another strain accumulated homoserine and threonine (Teas, Horowitz & Fling, 1948). During studies to ascertain whether strains of Coprinus accumulated methionine precursors, it was observed that cystathionine was formed in large amounts during growth of the wild type with methionine.

METHODS

Cultures were grown from stocks of *Coprinus lagopus* (Lewis, 1961) using H1 wild type. A spore suspension was prepared from slant cultures and an inoculum of 10^4 to 10^6 oidia used.

Growth. Cultures were grown in conical flasks, and incubated on a reciprocating shaker at 37° for 6–8 days.

Medium. Minimal medium contained per litre: ammonium tartrate, 0·5 g.; KH_2PO_4, 1·0 g.; Na_2HPO_4, 2·25 g.; Na_2SO_4, 0·29 g.; $MgSO_4.7H_2O$, 5·0 g.; glucose, 5·0 g.; asparagine, 4 mg.; thiamine HCl, 40 μg. For the experiments measuring sulphate incorporation, a low-sulphate medium was used in which Na_2SO_4 was omitted and the magnesium salt replaced by $MgSO_4.7H_2O$, 0·375 g. and $MgCl_2.6H_2O$, 0·103 g.

Harvest of mycelium and separation of amino acids. The mycelium was collected on a sintered funnel, washed with water, blotted, weighed and a sample taken for dry weight determination. The damp mycelium was broken up by grinding under liquid nitrogen and the free amino acids extracted with 80% (v/v) ethanol in water. The mycelial residue was dried and hydrolysed with 6 N-HCl for 16 hr at 125° in sealed tubes to liberate the protein amino acids. The filtrate (medium) and ethanol extract were each concentrated by evaporation *in vacuo* in a rotary evaporator. The extract was finally taken to a small volume, acidified with acetic acid, kept overnight at 1°, then the fat removed by filtration through an asbestos pad until the filtrate was clear. The pH was adjusted to below 5, and the extract prepared for chromatography by passing it through a column of Zeo–Karb 225, 50–100 mesh, in the hydrogen form. The amino acids were eluted with 4 N-NH_3 solution, the ammonia removed from the eluate by rotary evaporation and the amino acid residue taken up in 5 ml. 10% (v/v) *iso*-propanol. The medium was treated as above, with the omission of the defatting stage, but the hydrolysate residue was taken up directly in 10% (v/v) *iso*-propanol, after rotary evaporation to remove the HCl.

Chromatography. Chromatograms were run overnight on Whatman no. 3 MM paper: 31·5 × 33 cm. for ascending runs, and 22 × 50 cm. for descending. The following solvents, prepared fresh, were used: 1) *n*-butanol + acetic acid + water (12 + 3 + 5 by vol.), ascending. 2) phenol + ammonia + water, prepared by adding 60 ml. of water to 250 g. of phenol; just before chromatography 3 ml. NH_3 soln. (sp.gr. 0·88) and 3 ml. 2 mM-EDTA were added; ascending. 3) *n*-butanol + ethanol + water (2 + 2 + 1 by vol.), descending. 4) *tert.*-butanol + formic acid + water (14 + 3 + 3 by vol.), descending. To locate the amino acids, papers were dipped in 0·2% ninhydrin in acetone, with a trace of EDTA. Colours were allowed to develop in the cold for several hours, then at 100° for 5 min. Papers were finally sprayed with 250 mM-$NiSO_4$ to render the spots permanent. In routine examination of amino acid samples, one-dimensional chromatograms in solvent 1, and two-dimensional chromatograms in solvent 1 followed by solvent 2 were run. Differences in the qualitative pattern of amino acids were noted and any large quantitative differences estimated visually. Solvents 3 and 4 were used for the special separation of cystathionine from cysteine, cystine, and cysteic acid.

The suspected cystathionine was isolated quantitatively from the bulk of the amino acids by running a streak of the sample in solvent 1. Two marker spots

located the position of the appropriate band, which was cut out and eluted by shaking with 0·1 N-HCl in 10% (v/v) *iso*propanol. This fraction also included cysteine and some basic amino acids.

Bioassay of cystathionine. Cystathionine was assayed by the growth response of *Escherichia coli* 122/33 as described by Rowbury (1962).

Measurement of radioactivity. In the liquid samples, 10 μl. samples were spread evenly over uniform ground glass disks and dried. The ^{35}S activity was determined by counting the discs, usually for 1000 sec., with the aid of the Geiger–Muller tube of a Scaler 1700 (Isotope Developments Ltd.). In some cases the individual amino acids were separated on a chromatogram and specific areas of the paper counted either by scanning with a Geiger–Muller tube (Ratemeter Unit, type 1355B, Fleming Radio Developments Ltd.), or by cutting out, eluting the spots and counting samples from the liquid eluate on the ground glass discs.

Dry-weight determination. Mycelium was dried to constant weight over phosphorus pentoxide, at 40° and under vacuum.

RESULTS

Incorporation of ^{35}S from inorganic ^{35}S-sulphate into sulphur amino acids in wild type grown on methionine-supplemented medium

Cultures were grown in 1 l. flasks in 200 ml. of 'low-sulphate' medium, with approximately 40 μc. of carrier-free ^{35}S-sulphate added to each flask. Methionine (2 mM) was included in the medium, but omitted from control flasks. When harvesting, the mycelium was washed until washings were ^{35}S-free, to ensure no contamination of the mycelial fractions. The amino acid extracts and protein

Table 1. *Uptake and incorporation of ^{35}S-sulphate from the medium*

Figures show counts above background per 1000 sec. of 10 μl. samples from the liquid samples, volumes of which are shown in the final column. The extract counts are expressed per 100 mg. dry weight of mycelium. 50 mg. of dry residue was used for each of the hydrolysates. The background count was 135 counts/1000 sec. Values are given for two separate determinations with methionine-grown mycelium.

	Control	Growth with methionine		Volume (ml.)
Medium and washings after harvest	1352	1198	1516	200
Medium, amino acid fraction	471	591	479	5
Mycelium, ethanol extract	527	151	207	5
Mycelium, ethanol extract, amino acid fraction	139	30	48	5
Mycelium hydrolysate	1093	78	89	5

hydrolysates were prepared as described under METHODS, and the radioactivity of the amino acid fraction of the medium and the free and protein amino acids of the mycelium was measured. The two sets of cultures were treated in parallel, so that a direct comparison of radioactivity was made without necessitating a correction for natural decay of ^{35}S. The results are shown in Table 1.

There was apparently a marked decrease of ^{35}S incorporation into the mycelium amino acid fractions in cultures grown with methionine. In particular, there was a 92% decrease in label in the mycelium hydrolysate of these cultures. Counts of

specific spots *in situ* on a one-dimensional chromatogram of the hydrolysates are shown in Table 2. These show a decrease in incorporation into both methionine and cysteine: about 90% decrease of incorporation into cysteine, and 98% reduction of incorporation into methionine.

The free amino acids of the mycelium showed a 60–70% reduction in label content. In the amino acids of the medium, there was no significant difference in radioactivity in the control and methionine-grown cultures. This diminution in incorporation of ^{35}S into the amino acid and protein fractions of the mycelium in cultures grown with methionine indicates repression or end-product inhibition of the enzymes concerned in the synthesis of methionine. Furthermore, while the presence of methionine during the growth of wild-type cultures generally decreased

Table 2. *Radioactivity of methionine and cysteine in hydrolysates as measured in situ on a paper chromatogram*

Counts above background, detected with the chromatogram scanner, on an area 1 × 3 cm., for 10 min. The chromatogram was run in solvent 1. The background count was 132 counts in 10 min. 1 and 2 give the results of separate experiments.

	Control	Grown with methionine
Methionine	820	18
Cysteine (1)	230	26
(2)	396	44

the dry weight of mycelium, it markedly increased the concentration of free amino acids in the medium; thus, a visual estimate of the intensity of the ninhydrin spots suggested at least a 10-fold increase of most amino acids in the medium. The total amino acid content was not determined quantitatively because of the presence of methionine added to the initial medium. On the other hand, the content of free amino acids in the mycelium was less when grown in the presence of methionine, indicating that in spite of the overall stimulation of amino acid synthesis, the organism maintained its normal amino acid pool and excess amino acids were passed into the medium.

Accumulation of cystathionine by Coprinus lagopus

The most striking difference in the amino acid pattern, both in the medium and mycelial extracts, was the appearance of high concentrations of a compound X in the cultures grown with methionine. The position of the spot on two-dimensional chromatograms suggested that it was cystathionine, and on such chromatograms the ninhydrin spot was comparable in intensity with those of alanine and glutamic acid which are the amino acids usually present in the largest amounts. To establish conclusively that X was cystathionine, samples (isolated chromatographically, see METHODS) were chromatographed in four different solvents (Table 3). In two cases the samples were treated with H_2O_2 since cysteine and cystathionine are not separable in many solvents whereas the products of oxidation are. In each case X behaved like authentic cystathionine and this evidence, taken together with the fact that X supported the growth of *Escherichia coli* strain 122/33 (a cystathionine-requiring organism) may be taken as conclusive. For the bioassay of X, extracts of the mycelium were streaked on paper and after chromatography the appropriate band

was eluted and the cystathionine content determined. The results are given in Table 4 and show a large accumulation of cystathionine after growth with methionine, a moderate accumulation with added homocysteine, but no significant synthesis on medium with added cysteine plus homoserine. Cultures grown on minimal medium had only traces of cystathionine. When Coprinus was grown with methionine in the presence of ^{35}S-sulphate only traces of label appeared in the accumulated cystathionine.

Table 3. *Chromatographic identity of the accumulated compound X with cystathionine*

The positions of the spots are given in cm. from the origin. R_F values were not calculated as marker compounds were always run, and in some cases the solvent was allowed to run off the end of the paper. Oxidation was carried out by adding 2 vol. of 100 vol. hydrogen peroxide to the sample 20 min. before spotting.

		Standards		
	X	Cystathionine	Cysteine	Cysteic acid
Solvent 1. (1) No H_2O_2	3·6	3·5	3·4	3·9
(2) $+H_2O_2$	2·2	2·1	3·8	—
Solvent 2. (1) No H_2O_2	8·6	8·1	9·1	1·9
(2) $+H_2O_2$	5·7	5·2	1·7	—
Solvent 3. No H_2O_2	1·9	1·8	1·7	2·7
Solvent 4. No H_2O_2	6·9	7·0	5·4	13·0

Table 4. *Accumulation of cystathionine in wild-type Coprinus grown with methionine, homocysteine, or homoserine plus cysteine*

Cultures were grown in triplicate on minimal medium, supplemented as shown. The amino acids were extracted from the mycelium and the cystathionine separated by paper chromatography, and determined by bioassay. Concentrations are shown as μmole/100 mg. dry weight, and as % (w/w) of dry weight of mycelium.

	Accumulated cystathionine	
Supplement	μmole	%
None	0·276	0·061
2 mM-DL-methionine	6·82	1·52
2 mM-DL-homocysteine	1·33	0·29
mM-L-cysteine + mM-DL-homoserine	0·266	0·059

DISCUSSION

Part of the work described above was concerned with the incorporation of isotope from ^{35}S-sulphate by wild-type *Coprinus lagopus*. The results showed that the presence of methionine in the growth medium diminished the incorporation of ^{35}S into mycelial material insoluble in 80% ethanol (decreased to about 7·5%) and into the free amino acids of the mycelium (decreased to 30%). Incorporation of ^{35}S into the amino acids of the medium was not decreased by growth with methionine but as the amounts of these were increased by about 10-fold in the presence of methionine, there was probably a comparative diminution in incorporation (Table 1). Thus as Roberts *et al.* (1955) found for *Neurospora crassa* and *Escherichia coli*, methionine

decreased sulphate incorporation in Coprinus. When the proteins of the mycelium were hydrolysed, it was apparent (Table 2) that both cysteine and methionine contained smaller amounts of ^{35}S after growth with methionine although the decrease of methionine synthesis (to 2% of the control) was more marked than the decrease in cysteine synthesis (to 10% of the control). Inhibition of methionine formation (and therefore of ^{35}S incorporation) by methionine clearly suggests that synthesis in Coprinus is controlled by enzymic repression or feedback inhibition; both mechanisms of control are known to occur in this pathway in *E. coli* (Rowbury & Woods, 1961*a*, *b*; Rowbury, 1962). The effect of methionine on ^{35}S incorporation into cysteine may not be due to a direct inhibition. Pasternak (1961) and Ellis & Pasternak (1962) observed repression of sulphate activation by growth with cysteine in *E. coli* and a weaker repression effect with methionine. The latter effect they ascribed to formation of cysteine from methionine.

While studying the possible accumulation of sulphur-containing compounds, it was noticed that wild-type Coprinus accumulated cystathionine when grown in the presence of methionine but very little when grown without it (Table 4). The accumulation of this compound by auxotrophic mutants of *Neurospora crassa* has been observed (Horowitz, 1947); such mutants lacked cystathionase activity (Fischer, 1957) and it was concluded that the cystathionine had arisen via the 'normal' methionine synthesis pathway, i.e. from homoserine and cysteine. In support of this view Fischer (1957) showed that methionine inhibited this accumulation of cystathionine although one of the present authors (R. J. Rowbury, unpublished observations) has been unable to confirm this. Fischer (1957) observed that the slight cystathionine formation in wild-type Neurospora was increased by growth with methionine; but the extent of accumulation was small. The amount of cystathionine present in wild-type Coprinus was greatly increased (25-fold) by growth with methionine (Table 4) and this suggested that its synthesis was not via the 'normal' biosynthetic pathway. Furthermore, since ^{35}S from sulphate was not incorporated into the cystathionine this confirmed that the 'normal' pathway was not operative and since methionine was the only unlabelled sulphur compound present it is concluded that the cystathionine came from methionine by some pathway. It is presumed that the breakdown of the accumulated compound by cystathionase was prevented by methionine. Failure to accumulate cystathionine from added homoserine and cysteine may have been due to cystathionase activity and since cystathionase would also function during growth with homocysteine (although partial repression might occur due to synthesis of methionine from homocysteine) the accumulation of cystathionine in the homocysteine-supplemented medium may be indicative of an intermediary role of homocysteine in the synthesis of cystathionine from methionine. The system may be similar to that in Neurospora where cystathionine can be formed from homocysteine and serine (Delavier-Klutchko, 1963; R. J. Rowbury, unpublished observations).

Since methionine greatly decreased incorporation of ^{35}S from sulphate into cysteine a large part of the cysteine formed under such conditions must have arisen from methionine. This may have occurred by the partial breakdown of the accumulated cystathionine by a cystathionase enzyme of the type which occurs in animals (Matsuo & Greenberg, 1958) and in *Neurospora crassa* (Fischer, 1957). The inhibition of cysteine formation from sulphate would then probably be due, not to the direct

effect of methionine itself, but to the effect of the cysteine formed from it (as Ellis & Pasternak have suggested for *Escherichia coli*). The ability of organisms to convert methionine to cysteine may be a widespread property and it would be interesting to know whether the system is induced by methionine. The view that cysteine can arise from methionine in Coprinus via cystathionine formation is supported by the observation in this laboratory that ^{35}S-ethionine gives rise to cysteine and the process is associated with the accumulation of cystathionine (D. Lewis, personal communication).

The authors wish to thank Professor D. Lewis, F.R.S., for suggesting the problem and for his enthusiastic interest and advice during the work.

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J. gen. Microbiol. (1964), **36**, 341–358
Printed in Great Britain

O-Succinylhomoserine as an Intermediate in the Synthesis of Cystathionine by *Escherichia coli*

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(*Received* 25 *March* 1964)

SUMMARY

Further evidence that cystathionine is a precursor of methionine synthesis in *Escherichia coli* was obtained by showing its synthesis by cell-free extracts of an auxotroph which required homocysteine or methionine for growth. Succinate, as well as homoserine and cysteine, was an obligatory substrate and maximal synthesis was dependent on the further addition of glucose, adenosine triphosphate and coenzyme A. The reaction was further studied by using mixtures of extracts of auxotrophs of which each alone was unable to synthesize cystathionine. At least two enzymes were required: the first, present in one strain, formed a heat-stable intermediate from homoserine and succinate; while the second, present in the other strain, converted this intermediate to cystathionine in the presence of cysteine. The intermediate was formed enzymically from homoserine and succinyl-coenzyme A alone and was ninhydrin-positive; this suggested that it might be *O*-succinylhomoserine. This compound was prepared by reaction of succinyl chloride or succinic anhydride with homoserine in the presence of perchloric acid; its chromatographic and chemical properties were closely similar to those of the intermediate. The synthetic product, like that formed enzymically, gave cystathionine when incubated with cysteine and a cell-free extract of the relevant *E. coli* auxotroph. It is concluded that *O*-succinylhomoserine is an intermediate in the formation of cystathionine, and consequently of methionine by *E. coli*.

INTRODUCTION

The properties of the various auxotrophic strains of *Escherichia coli* which give a growth response with methionine strongly suggest that this amino acid arises from homoserine + cysteine with the intermediate formation of cystathionine and homocysteine (see review by Davis, 1955). This role of homocysteine has been amply confirmed with suspensions of intact organisms and with cell-free enzyme preparations (Gibson & Woods, 1960; Guest, Helleiner, Cross & Woods, 1960). In the case of cystathionine closer investigation has given apparently contradictory results. It was established that extracts of many strains of *E. coli* contained an enzyme, cystathionase, which catalysed the formation of homocysteine from cystathionine (Wijesundera & Woods, 1953, 1962); the probable physiological significance of this enzyme in methionine synthesis was emphasized by the fact that its formation was repressed by methionine, the ultimate product of the reaction sequence (Rowbury & Woods, 1961*b*, 1964). On the other hand, the work of Bolton, Cowie & Sands (1952) with growing cultures of *E. coli* cast doubt on cystathionine as an inter-

mediate since it did not, as would be expected, decrease the incorporation into bacterial protein of [^{35}S]-sulphate added to the medium. While these results might be explained either by a relative impermeability of the organism to cystathionine or by lack of equilibration of free cystathionine with enzyme-bound cystathionine within the organism, the case for cystathionine would clearly be strengthened if the organism were found to contain enzymes for its synthesis from the presumed precursors as well as the enzyme already demonstrated for its conversion to the next intermediate.

An auxotrophic strain of *Escherichia coli* known to lack cystathionase was chosen initially for the investigation since it would not be able to degrade any cystathionine formed. The trial of succinate as co-substrate with homoserine and cysteine was prompted by a brief report (Davis, Kornberg, Nagler, Miller & Mingioli, 1959) that certain succinate-requiring mutants of *E. coli* grew in absence of succinate when provided with a mixture of methionine + lysine + threonine. This suggested that succinate might be a reactant at some stage of the synthesis of each of these amino acids. Furthermore, correlation of the nutritional and genetic properties of methionine auxotrophs of *Neurospora crassa* (Buss, 1944) and *Salmonella typhimurium* (Smith, 1961) indicated that more than one enzyme was concerned in cystathionine synthesis from homoserine and cysteine. Brief reports of part of the present work were given by Rowbury (1961, 1962*b*).

METHODS

Organisms and enzyme extracts. All the auxotrophic strains of *Escherichia coli* used in this work were maintained on slopes of Oxoid nutrient agar, subcultured monthly and stored at 4° after incubation for 18 hr at 37°. Strain 26/18 responded to homocysteine or methionine and the following strains responded also to cystathionine: 122/33, 2/2, 7/9, 6/5, 7/17 G, 2/17, 8/15. Strain B 184 grew with homoserine or with methionine + threonine. Strain 122/33, derived from *E. coli* W, was provided by Dr B. D. Davis and B 184 (from *E. coli* B) by Dr J. S. Gots; the remaining strains were isolated in this laboratory after ultraviolet (u.v.)-irradiation of the parent strain *E. coli* 518 (ATCC 9723).

Organisms were grown on the glucose lactate salts medium (*GL*) of Guest *et al.* (1960) supplemented with DL-homocysteine (0·25 mM). Conditions of growth and harvesting and the methods used for the preparation and subsequent treatment of cell-free extracts were as described by Rowbury & Woods (1961*a*).

Study of cystathionine formation. The basal reaction mixture (solution *A*) normally contained (mM in 3 ml. of 80 mM-potassium phosphate buffer, pH 7·5): DL-homoserine, 5; L-cysteine, 3·3; sodium succinate, 50; adenosine triphosphate, 3·3; glucose, 6·6; coenzyme A, 0·06. After addition of the enzyme preparation (equivalent to 3·3 mg. protein/ml.) the tubes were incubated in air at 37° for 3–4 hr. The reaction was terminated by heating at 100° for 3 min. and cystathionine assayed in the supernatant fluid obtained after centrifugation.

Microbiological assays. A strain of *Escherichia coli* (122/33) giving a growth response with cystathionine was used for the estimation of this amino acid. Medium *GL* was used, with DL-cystathionine as standard. The tubes (150 × 20 mm. containing 4 ml. medium) were incubated in a sloped position in air at 37° for 40 hr and the

amount of growth was assessed in a Spekker Photoelectric Absorptiometer H760 (Hilger & Watts, London) with 0·5 cm. light cells. The shape of the curve relating dosage to response to pure cystathionine (Fig. 1) was similar with typical experimental samples. The assay organism responds also to homocysteine and methionine, but in experiments on cystathionine formation by extracts of *E. coli* 26/18 these amino acids were shown to be absent by independent assays (Table 1). In experiments in which extracts of *E. coli* strains 7/9 and 2/2 were used as enzyme source it was possible that any cystathionine formed would be converted wholly or in part to homocysteine since both these organisms contain cystathionase. The assay was not, however, invalidated since homocysteine, and mixtures of homocysteine + cystathionine give quantitatively the same response as cystathionine itself (Fig. 1). Furthermore, methionine is not formed from homocysteine by extracts of those organisms under the conditions of the test for cystathionine synthesis. The results are therefore expressed throughout in terms of cystathionine.

The procedure for the estimation of homoserine and homocysteine was similar to that for cystathionine except that the assay organisms were, respectively, *Escherichia coli* B 184 and *E. coli* 26/18. Methionine was assayed with *Streptococcus equinus* P 60 as described by Gibson & Woods (1960).

Estimation of succinic acid. This was titrated with 0·01 M-barium hydroxide after evaporating to dryness the fraction eluted from columns of Dowex-1 resin with 6 N-formic acid.

Chromatography on paper. For the separation and identification of cystathionine, chromatograms were developed on Whatman no. 1 paper with *n*-butanol + acetic acid + water (2 + 1 + 1, by vol.). When cystathionine was to be assayed, the chromatograms were dried for 2 hr at 100° and the relevant regions eluted with water. Homoserine and *O*-succinylhomoserine were separated from one another either by the above solvent mixture or by *n*-butanol + propionic acid + water (47 + 22 + 31, by vol.) Amino acids were detected by spraying with ninhydrin (0·2%, w/v, in *n*-butanol saturated with water) and heating at 80° for 10 min. Succinic acid was detected with bromocresol green (0·04% solution in water adjusted to pH 7·5.)

In some experiments with isotopically-labelled succinate the chromatograms were either scanned directly or the material eluted by water from specific regions counted after spreading on metal or glass discs. A mica end-window Geiger–Müller tube (General Electric Co. type 2B2) was used in conjunction with a Scaler 1700 of Isotope Developments Ltd. Reading, Berks.

Column chromatography. In the earlier part of the work the resin used was Dowex-50 (H form, ×4, 200–400 mesh) and the procedure that of Wall (1953). Experimental samples in N-HCl were placed on 30 × 0·9 cm. columns and the amino acids eluted with N-HCl, separate fractions being collected. Smaller columns were used (5 × 0·9 cm.) when the object was only to free the samples from succinate; these were applied in neutral solution and, after washing the column thoroughly to remove succinate, the mixed amino acids were removed with N-HCl.

In other experiments, Dowex-1 resin (acetate form, ×8, 200–400 mesh) was used. Samples in aqueous solution were applied to a previously washed column (18 × 0·9 cm.) that was then eluted with 15 ml. water followed by 0·2 N-acetic acid.

Fractions of eluates from both types of column were assayed for amino-N by using the modified ninhydrin reagent of Moore & Stein (1954); HCl was removed

from fractions to be tested for biological activity by evaporating to dryness in a small rotary evaporator.

Chemicals. Coenzyme A was a product of the Sigma Chemical Company (St Louis, Mo., U.S.A.) and a solution of succinyl-coenzyme A was prepared from it by the method of Simon & Shemin (1953) immediately before use. Succinic anhydride (0·1 M; 0·25 ml.) was added to the coenzyme (15 mg. in 2 ml. of water at 0°) and the mixture stood at 0° for 20 min. after adjustment to pH 7. [$^{14}C_{2,3}$]-Succinic acid was obtained from the Radiochemical Centre, Amersham, Bucks.

The sources of other special chemicals were as described by Rowbury & Woods (1961*a*, 1964).

Table 1. *Differential microbiological assay of the products formed by cell-free extracts of Escherichia coli 26/18*

Ultrasonic extract (equiv. 3·3 mg. protein/ml.) was incubated for 4 hr in solution *A* with the stated omissions. A control in which the complete reaction mixture was heated for 3 min. at 100° before incubation gave values not exceeding 0·05 μmole in any case; the appropriate value has been deducted from the results given.

	Assay organism		
	E. coli 122/33	*E. coli* 26/18	*Streptococcus equinus* P 60
	Product assayed		
	Cystathionine + homocysteine + methionine	Homocysteine + methionine	Methionine
Omissions from solution *A*	Product formed (μmole)		
None	1·39	0·04	0·00
Homoserine	0·11	0·06	0·00
Cysteine	0·15	0·03	0·01

RESULTS

Cystathionine formation by Escherichia coli 26/18

This auxotroph lacks the cystathionase enzyme and therefore responds only to homocysteine or methionine; it was assumed that it would retain the ability to synthesize cystathionine. Unsuccessful attempts had been made earlier to demonstrate this with intact organisms and with cell-free preparations and the indicated precursors (homoserine + cysteine) in the presence of various mixtures of possible energy sources and cofactors (e.g. glucose, adenosine triphosphate (ATP), coenzyme A, pyridoxal phosphate). However, the further addition of succinate to unpurified ultrasonic extracts incubated in a mixture of all the above substances led to the production of material which supported the growth of *E. coli* 122/33, an auxotroph which responds to cystathionine as well as to homocysteine and methionine. Concurrent assay of the reaction products with *Streptococcus equinus* P 60 (which responds only to methionine) and *E. coli* 26/18 itself (which responds only to homocysteine and methionine) showed that very little methionine or homocysteine was

present (Table 1); they accounted, at most, for only about 5% of the growth response given by *E. coli* 122/33 and cystathionine was therefore indicated as the major product. The reaction products were also assayed with this organism after chromatography on paper with *n*-butanol + acetic acid + water and eluting separate regions of the chromatogram (Fig. 2). The active reaction product had a similar R_F value to cystathionine, which was clearly distinguishable from those for methionine and homocysteine. Homocysteine was partly oxidized to homocystine and gave two active regions on the chromatogram. It was concluded provisionally that the product was cystathionine.

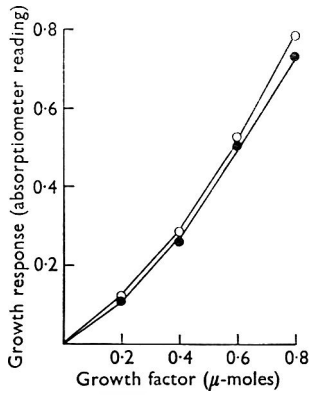

Fig. 1

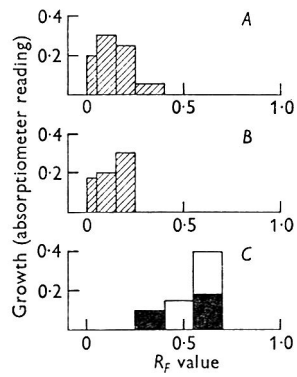

Fig. 2

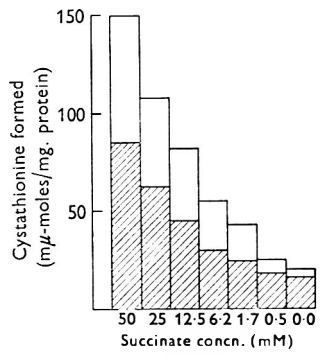

Fig. 3

Fig. 1. Growth response of Escherichia coli 122/33 to cystathionine (○) and homocysteine (●).

Fig. 2. Identification of cystathionine by paper chromatography followed by microbiological response to Escherichia coli 122/33. Extract of *E. coli* 26/18 was incubated for 3 hr in solution *A* and the de-proteinized products chromatographed with *n*-butanol + acetic acid + water. After 20 hr, regions of the chromatogram were eluted with water and assayed with *E. coli* 122/33 (*A*). Pure specimens of cystathionine (*B*) and homocysteine and methionine (*C*) were given the same overall treatment.

Fig. 3. Effect of succinate and coenzyme A on cystathionine formation. Ultrasonic extract of Escherichia coli 26/18 was incubated for 4 hr in solution *A* (with and without coenzyme A) containing the stated concentrations of succinate. With coenzyme A, open; without coenzyme A, hatched.

Requirements for cystathionine formation. Optimal synthesis of cystathionine occurred with dialysed, though otherwise unpurified, ultrasonic extracts of the organism in a reaction mixture (solution *A*, see Methods section) containing homoserine, cysteine, succinate, glucose, ATP and coenzyme A. Synthesis was decreased by 80–90% when homoserine and/or cysteine were omitted (Table 2); the residual formation may have been due to sources of these amino acids in the enzyme preparation. A small amount of cystathionine was formed when homoserine was replaced by aspartic acid, which is known to be its precursor in *Escherichia coli* (Hirsch & Cohen, 1954).

Omission of succinate also decreased cystathionine formation by 80–90% (Table 2; Fig. 3). Synthesis increased with increasing succinate concentration up to 50 mM, which was the highest value tested (Fig. 3). Of a number of substances structurally or metabolically related to succinate, only α-oxoglutarate, which had

about half the activity of succinate, replaced it to any significant extent; fumarate, malate, citrate, acetate and acetyl-coenzyme A were essentially inactive. The effect of omitting coenzyme A from the reaction mixture was tested over a range of concentrations of succinate (Fig. 3); in each case cystathionine formation was decreased about twofold. The effect of coenzyme A was only slightly sharpened by treatment of the enzyme preparation with Dowex-1 resin; it seems probable that bound forms were present. The results suggest that succinate may act in cystathionine formation after prior conversion to succinyl-coenzyme A.

Table 2. *Cystathionine formation by extracts of Escherichia coli 26/18*

Ultrasonic extract was incubated for 3 hr in solution *A*, modified as indicated; aspartate and pyridoxal phosphate were 5·0 and 0·5 mM, respectively. —, Indicates no addition or omission.

Modifications to solution *A*		Cystathionine formed (μm-mole/mg. protein)
Omitted	Added	
—	—	190
Cysteine + homoserine	—	10
Cysteine	—	22
Homoserine	—	18
Homoserine	aspartate	50
Succinate	—	25
Coenzyme A	—	100
Glucose	—	42
ATP	—	31
—	pyridoxal phosphate	195

Optimal synthesis of cystathionine also required the presence of glucose (replaceable by hexose diphosphate) + ATP, presumably as components of an energy-generating system (Table 2). It is unlikely therefore that succinate acts as a source of energy, and a more specific role is indicated.

Pyridoxal phosphate had no effect on cystathionine formation under the usual test conditions nor when the dialysed enzymic extracts had been further treated with Dowex-1 resin. If it is the prosthetic group of any of the enzymes concerned in the present amino acid transformations it is clear that it is not readily dissociated.

Kinetics. The reaction was optimal at pH 7·5, cystathionine formation being decreased to 40 % and 80 % of the optimum value at pH 6·9 and 8·0, respectively. With all the required accessory substances present there was a linear relationship between time and amount of product formed up to 2 hr; appreciable formation occurred for a further 3 hr.

Cystathionine formation by mixed extracts of cystathionine-requiring mutants

The results so far reported suggested that there is more than one enzymic step in the overall formation of cystathionine from homoserine, cysteine and succinate. It is possible that auxotrophs which fail to synthesize cystathionine might lack different enzymes, and consequently that a mixture of extracts from appropriate auxotrophs might achieve the synthesis, one extract providing the enzyme or enzymes missing from the other. This proved to be the case (Table 3); in a survey of six auxotrophs of *Escherichia coli* of this type the extract of one of them (strain 2/2)

was effective in admixture with extract of any of three other strains (7/9, 6/5 and 7/17 G), but not with the other two. As expected all the individual extracts were inactive. Since all these auxotrophs contain cystathionase, the product assayed was probably a mixture of cystathionine and homocysteine. For reasons stated in the Methods section the results may legitimately be given in terms of cystathionine.

Table 3. *Formation of cystathionine by mixtures of extracts of various cystathionine-requiring auxotrophs of Escherichia coli*

Ultrasonic extract (equiv. 3·3 mg. protein/ml.) of the stated auxotroph was incubated for 4 hr in solution *A* with and without extract of auxotroph 2/2 (equiv. 3·3 mg. protein/ml.)

Extract derived from *E. coli* strain no.	Cystathionine formed (*μ*m-mole/mg. protein) with extract of *E. coli* strain 2/2	
	Absent	Present
2/2	—	9
7/17 G	5	104
6/5	5	100
2/17	6	6
7/9	8	126
8/15	5	10

Table 4. *Cystathionine formation by a mixture of extracts of Escherichia coli strains 7/9 and 2/2*

Ultrasonic extracts (equiv. 5 mg. protein of each strain) were incubated together in 3 ml. of solution *A* with the stated omissions. Pyridoxal phosphate, 0·5 mM.

Omission from solution *A*	Cystathionine formed (*μ*m-mole/mg. protein)
None	160
Homoserine	12
Cysteine	15
Succinate	20
Glucose + ATP	15
None (pyridoxal phosphate added)	160

The active mixture of extracts from strains 2/2 and 7/9 was selected for detailed investigation. Homoserine, cysteine, succinate and an energy source were required for significant formation of cystathionine (Table 4), thus confirming that the reaction under study was similar to that catalysed by the competent strain 26/18. Pyridoxal phosphate was again without effect. Information about the probable order of action of the enzyme or enzymes present in each extract was obtained from experiments in which only one extract was added initially and the other about half-way through the period of incubation (Table 5). It is clear that strain 7/9 acted first and that an intermediate compound accumulated which was converted to cystathionine either by enzymes of strain 2/2 alone or by these + other enzymes from strain 7/9.

Nature of the first reaction. The particular substrates required for the two steps in cystathionine formation were determined in further two-part experiments in which

extract of strain 7/9 was always used in the first incubation but various mixtures of the three substrates were added either during the first or second period of incubation (Table 6). Maximum synthesis of cystathionine was obtained when homoserine + succinate + extract of strain 7/9 were present initially and cysteine + extract of strain 2/2 was added later. Under all other conditions, except the control in which all three substrates were present from the beginning, synthesis was low. These results strongly suggest that an intermediate formed from homoserine and succinate by the enzymes of strain 7/9 was converted to cystathionine in the presence of cysteine by an enzyme or enzymes from strain 2/2.

Table 5. *Cystathionine formation by mixed cell-free extracts: sequence of action*

Ultrasonic extracts of *Escherichia coli* strains 7/9 and 2/2 (each equiv. 5 mg. protein/3 ml.) were added as indicated to solution *A*, either initially or after incubation for 2·5 hr; total time of incubation 4 hr.

Source of extract added		Cystathionine formed (μm-mole/mg. protein)
Initially	After 2·5 hr	
Both strains	—	124
Strain 7/9	Strain 2/2	135
Strain 2/2	Strain 7/9	46
Nil	Both strains	21

Table 6. *Cystathionine formation by mixed extracts: order of utilization of the substrates*

Homoserine, cysteine and succinate were added as indicated to the other components of the solution *A*, either initially or after incubation for 2·5 hr. Extract of *Escherichia coli* strain 7/9 was added initially and that of *E. coli* strain 2/2 at 2·5 hr; each were equiv. 5 mg. protein/3 ml. Total time of incubation 4 hr.

Substrate added		Cystathionine formed (μm-mole/mg. protein)
Initially	Added 2·5 hr	
Homoserine + succinate + cysteine	—	124
Homoserine + succinate	Cysteine	203
Succinate	Homoserine + cysteine	57
Cysteine + succinate	Homoserine	51
Homoserine	Cysteine + succinate	40
Cysteine	Homoserine + succinate	20
Homoserine + cysteine	Succinate	34
Nil	Homoserine + succinate + cysteine	20

Heat-treatment of the reaction mixture at the end of the first incubation to inactivate the enzymes present in extract of strain 7/9 did not significantly decrease the yield of cystathionine on further addition of cysteine + extract of strain 2/2 (Table 7). No enzyme uniquely present in strain 7/9 was therefore required for the second reaction in addition to enzymes present in strain 2/2. The intermediate compound was moderately stable to heat; similar results were obtained with 2 min. at 100°, though longer periods at this temperature were detrimental.

Nature of the intermediate compound

Preparations of the intermediate were obtained by incubating ultrasonic extracts of *Escherichia coli* 7/9 in the usual reaction mixture (solution *A*) but without cysteine. The intermediate was detected and assayed biologically by measuring cystathionine formation when samples were incubated with ultrasonic extracts of strain 2/2 and a buffered reaction mixture containing cysteine + glucose + ATP, i.e. solution *A* from which homoserine, succinate and coenzyme A had been omitted.

Table 7. *Effect of mild heat-treatment on the intermediate compound*

Extract of *Escherichia coli* strain 7/9 was incubated with solution *A* (cysteine omitted) for 2·5 hr; extract of *E. coli* strain 2/2 + cysteine were then added and incubation continued for a further 1·5 hr. The reaction mixtures were heated as indicated.

Heat-treatment	Cystathionine formed (μm-mole/mg. protein)
None	112
55° for 2 min. initially	14
55° for 2 min. at 2·5 hr.	96
55° for 10 min. initially	7
55° for 10 min. at 2·5 hr.	98

Separation by chromatography. Paper chromatograms of samples of the untreated reaction products were developed with *n*-butanol + propionic acid + water solvent; comparison with the initial reaction mixture showed the presence of a new ninhydrin-positive compound of R_F value 0·35 as compared with 0·27 for homoserine. The colour obtained with ninhydrin on paper was initially yellow-brown with this new compound. It was not present when either homoserine or succinate was omitted from the original reaction mixture. Several amino acids metabolically related to homoserine or succinate were clearly separated from this material on the chromatogram.

The intermediate compound was also separated from the reaction mixture by chromatography on columns of Dowex-50 resin. Unchanged succinate was not bound and was washed through with water. Homoserine and the presumed intermediate were eluted by N-HCl; an almost complete separation was obtained on a 30 cm. column (Fig. 4). The colour given by ninhydrin in tests in solution was similar to that of homoserine (which was used as standard in the amino-N determinations) and not the yellow brown obtained in the test on dried paper. Pooled fractions covering the regions of the two peaks were evaporated to dryness, redissolved and tested separately for conversion to cystathionine in the presence of the enzyme preparation from strain 2/2; only the second component eluted was active in this sense and chromatography on paper showed that only it contained the ninhydrin-positive material of R_F value 0·35. It was concluded that this material was the intermediate compound.

Stability to acid and alkali. Unpurified reaction mixtures containing the intermediate compound were adjusted to various pH values, incubated at 37° for 5 min. and re-adjusted to pH 7·5. The amount of intermediate which survived was assayed

enzymically with extract of strain 2/2 as described above (Fig. 5). The intermediate compound was highly unstable above pH 8 and was almost completely destroyed at pH 11.

Although the intermediate was relatively stable to mild acid treatment at 37° (Fig. 5), all activity was lost by heating for 2 hr with N-HCl at 100°. Material purified by chromatography on Dowex-50 resin was used in these experiments. The presence of homoserine and succinate in the products of acid-treatment (presumably hydrolysis) was shown by paper chromatography; homoserine was also detected by the growth response of *Escherichia coli* B 184, a homoserine auxotroph.

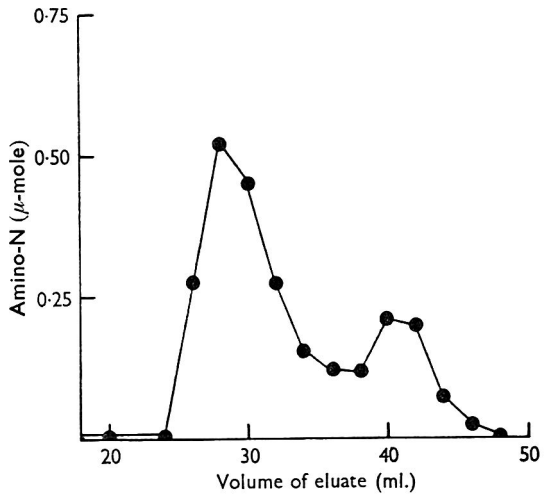

Fig. 4

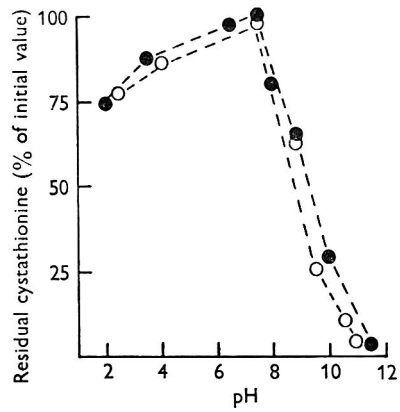

Fig. 5

Fig. 4. Chromatographic separation of the intermediate from homoserine. Extract of Escherichia coli 7/9 was incubated for 4 hr in solution *A* (omitting cysteine). The deproteinized products were concentrated *in vacuo* and chromatographed on Dowex-50 resin as described in the Methods section. Fractions of the eluate were assayed for amino-N.

Fig. 5. The effect of treatment with mild acid and alkali on the intermediate and its synthetic counterpart. Unpurified reaction mixtures containing the intermediate were incubated at the stated pH value for 5 min. at 37°, readjusted to pH 7·5 and the residual intermediate assayed (as cystathionine) after incubation with cysteine and extract of Escherichia coli 2/2. A synthetic preparation of *O*-succinylhomoserine was similarly treated. ●, intermediate formed enzymically; ○, synthetic product.

Incorporation of radioactive succinate. The results so far recorded suggested that the intermediate was a derivative of succinic acid. Confirmation of this was sought by experiments in which enzymic synthesis of the intermediate took place in the presence of [$^{14}C_{2,3}$]-succinate, which was added (5 μC/3 ml.) to the reaction mixture. The unpurified products of the reaction were placed on a short column of Dowex-50 resin and unchanged succinate removed by passing water through until the washings showed no radioactivity. Amino acids, which include the intermediate compound, were eluted with N-HCl and samples were assayed for content of ^{14}C; five times as much isotope was incorporated when homoserine was present in the initial reaction mixture than when it was absent. Samples of the eluate chromatographed on paper

(solvent, *n*-butanol + propionic acid + water) showed a radioactive region with an R_F value about 0·35 (Fig. 6), corresponding with the R_F value of the intermediate compound with this solvent. The small amount of radioactivity of R_F 0·7 was due to succinate which may have been formed by decomposition of some of the intermediate, or have escaped removal during the treatment with Dowex resin. When the amino acid fraction was treated with hot acid before running the chromatogram, the bulk of the radioactivity now appeared at R_F 0·7 (corresponding with succinate) and only traces at R_F 0·35 (Fig. 6).

In one experiment a comparison was made in molar terms between the amount of homoserine used, the amount of intermediate compound formed (in terms of cystathionine formed enzymically from it) and the amount of succinate incorporated into the amino acid fraction. The concentration of DL-homoserine in the usual reaction mixture was decreased to 1·7 mM and the succinate contained 5 μC of the isotopically labelled compound. The amount of homoserine present was assayed microbiologically before and after incubation for 2·5 hr; other samples were used for the enzymic assay of the intermediate. The amount of succinate incorporated was calculated from the radioactivity of the material eluted by N-HCl from a Dowex-50 column (washed to remove unchanged succinate) which had been loaded with the remainder of the incubated reaction mixture. The amount of homoserine metabolized was 0·58 μmole; 0·54 μmole of the intermediate were formed and 0·49 μmole of succinate were incorporated. It was likely therefore that the intermediate contained one homoserine and one succinate residue; the slightly low result obtained for succinate may have resulted from losses during the resin treatment since the other two values were derived from direct assays of the untreated reaction products.

The role of succinyl-coenzyme A

Coenzyme A increased the overall synthesis of cystathionine from homoserine + succinate + cysteine by enzyme preparations of *Escherichia coli* 26/18 (Fig. 3). Succinate was found above to be required only in the reaction leading to the formation of the intermediate compound from homoserine; it was possible therefore that it was first converted to succinyl-coenzyme A by a succinyl-coenzyme A synthetase also present in the extracts of strain 7/9 which formed the intermediate. In the following experiments formation of the intermediate was studied with an extract of *E. coli* strain 7/9 that had been stored at −15° for 30 days and had lost 60% of its activity.

Addition to the reaction mixture of a purified preparation of succinyl-coenzyme A synthetase derived from *Rhodopseudomonas spheroides* (Burnham, 1963; kindly provided by Dr B. F. Burnham) increased twofold the formation of the intermediate. It is likely therefore that *Escherichia coli* contains this enzyme and that it decays more rapidly than the other enzymes concerned in cystathionine synthesis. Succinyl-coenzyme A prepared chemically was tested by the usual two-stage incubation method in which the intermediate compound formed by extract of *E. coli* strain 7/9 was later converted to cystathionine in the presence of cysteine + extract of strain 2/2. Four times as much cystathionine was formed with succinyl-coenzyme A than with succinate + coenzyme A (compare Expt. 1 and 2, Table 8). Furthermore, the addition of glucose + ATP could be deferred until the second

stage of the incubation without seriously diminishing the amount of cystathionine finally produced (Expt. 3). This means that although an energy source was required for the reaction between the intermediate and cysteine (compare Expt. 3 and 4) it was not required for that between homoserine and succinyl-coenzyme A to form the intermediate. The latter reaction proceeds satisfactorily in a buffered system containing only enzymic extract, homoserine and succinyl-coenzyme A (Expt. 3); the presence of the intermediate under these conditions was confirmed by paper chromatography.

Table 8. *The role of succinyl-coenzyme A in cystathionine formation*

An 'aged' extract of *Escherichia coli* strain 7/9 (see text) was incubated for 2·5 hr in buffered homoserine with the stated additions; succinyl-coenzyme A was 1·7 mM and all other concentrations as in solution *A*. The reaction mixtures were then heated for 2 min. at 100° and incubation continued for a further 1·5 hr after adding extract of *E. coli* strain 2/2, cysteine and any other substance indicated. CoA, coenzyme A; succinylCoA, succinyl-coenzyme A; —, no addition.

Expt.	Substance added: Initially	Substance added: At 2·5 hr	Cystathionine formed (μm-mole/mg. protein)
1	Succinate + CoA + glucose + ATP	—	40
2	SuccinylCoA + glucose + ATP	—	160
3	SuccinylCoA	Glucose, ATP	137
4	SuccinylCoA	—	52
5	Succinate + CoA	—	10
6	Glucose + ATP	—	6

Experiments with a synthetic succinyl derivative of homoserine

The results recorded in the earlier sections indicated that the intermediate in cystathionine synthesis in *Escherichia coli* was an alkali-labile succinyl derivative of homoserine in which the amino group was still free. The obvious possibility was *O*-succinylhomoserine; no synthesis of this compound had been reported.

Chemical synthesis. No *O*-succinyl derivative of any hydroxyamino acid has so far been described; the synthesis of *O*-acetyl derivatives of serine, threonine, hydroxyproline and tyrosine by reaction of the amino acid with acetic anhydride in glacial acetic acid in the presence of perchloric acid (to prevent *N*-acetylation) was reported by Sakami & Toennies (1942). This method was later used for the preparation of *O*-acetylhomoserine (Matsuo, Rothstein & Greenberg, 1956; Grobbelaar & Steward, 1958). Synthetic material, corresponding in every way to the intermediate formed enzymically, was obtained from homoserine by using succinyl chloride or succinic anhydride as acyl donor and in the presence of perchloric acid to prevent acylation of the amino group. (*a*) DL-Homoserine (15 mg.) and succinyl chloride (0·11 ml.) were incubated in dioxane containing 0·5 M-perchloric acid (1 ml.) for 30 min. at 40°. (*b*) DL-Homoserine (15 mg.) was mixed with succinic anhydride (600 mg.) and the mixture melted; after adding perchloric acid to a final concentration of about 0·5 M, it was kept molten (above 120°) for 10 min. In both cases the products were neutralized with potassium carbonate solution and the precipitated potassium perchlorate removed by centrifugation. The supernatant fluids were evaporated to dryness (which removed dioxane when present) and the residues taken up in water.

Chromatographic properties and purification. The likely impurities (succinic acid, homoserine) were removed by the use of Dowex-1 resin. Homoserine was not retained by the resin and was washed through with water. The succinyl derivative, though not succinic acid, was eluted by 0·2 N-acetic acid; succinic acid was eluted by 6 N-formic acid. The separation of homoserine from its succinyl derivative is illustrated in Fig. 7 for a mixture of the two substances. The intermediate compound formed enzymically appeared in the same fractions of the acetic acid eluate (Fig. 7).

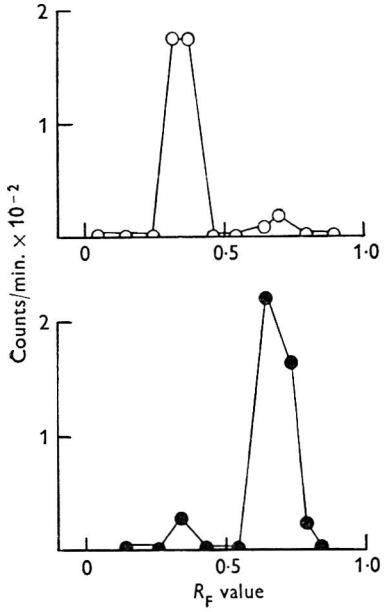

Fig. 6

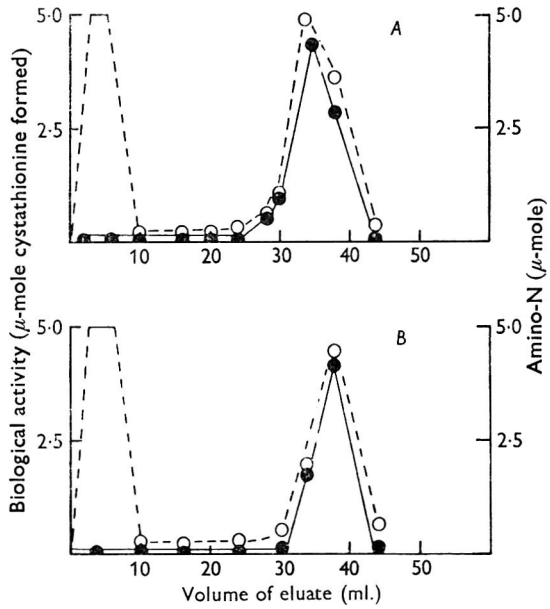

Fig. 7

Fig. 6. Incorporation of radioactive succinate into the intermediate. Reaction mixtures containing the intermediate were obtained by incubating extract of Escherichia coli 7/9 in solution *A* (minus cysteine) containing 5 μC [$^{14}C_{2,3}$]-succinate. The material was chromatographed on paper (solvent, *n*-butanol + propionic acid + water) before (○) and after (●) heating with N-HCl for 2 hr at 100°. The chromatograms were scanned directly for radioactivity.

Fig. 7. Chromatography of the intermediate and its synthetic counterpart on Dowex-1 resin. Samples of synthetic *O*-succinylhomoserine (mixed with homoserine) and of enzymic reaction products containing the intermediate were chromatographed on Dowex-1 resin as described in the Methods section. Fractions of the eluate were assayed for amino-N (– –○– –) and for biological activity (—●—) in terms of enzymic production of cystathionine in the presence of cysteine. *A*, intermediate formed enzymically; *B*, synthetic *O*-succinylhomoserine.

The compounds formed chemically and enzymically gave identical R_F values when chromatographed on paper with two different solvent systems, R_F 0·35 with *n*-butanol + propionic acid + water and R_F 0·59 with *n*-butanol + acetic acid + water; the corresponding values for homoserine itself were 0·27 and 0·50. The two compounds also both gave a yellow-brown colour on heating on paper with ninhydrin; this changed to purple on standing.

Decomposition by acid and alkali. Synthetic material purified by chromato-

graphy on Dowex-1 resin was assayed for amino-N by the ninhydrin method, with DL-homoserine as standard. A portion was heated at 100° for 2 hr in N-HCl and fractions corresponding to homoserine and succinic acid obtained after treatment with Dowex-1 resin (see above). Homoserine was assayed microbiologically and succinic acid by titration; the latter was not identified specifically. The succinylhomoserine (21·2 μmole of amino-N) gave 22·0 μmole homoserine and 23·7 μmole of a dibasic acid, presumed to be succinic acid from its chromatographic behaviour and by analogy with the similar acidic product of hydrolysis of the natural intermediate which was shown by isotope technique to be succinic acid. Another portion of the same purified material was incubated at pH 11 for 5 min. at 37°. Chromatography on paper showed that the spot corresponding to the original material had disappeared, but that no homoserine or other ninhydrin-positive compound had been formed. Homoserine (10·5 μmole from 21·2 μmole amino-N of the original succinylhomoserine) was, however, formed on acid hydrolysis of the alkali-treated material.

Table 9. *Cystathionine formation from synthetic O-succinylhomoserine: effect of cysteine concentration*

A preparation of *O*-succinylhomoserine (1 mM), purified by chromatography on Dowex-1 resin, was incubated for 3 hr in phosphate buffer containing glucose + ATP (concentrations as in solution *A*) with extract of *Escherichia coli* strain 2/2 (protein, 5 mg./3 ml.) and L-cysteine at the concentrations stated.

Concn. of cysteine (mM)	Cystathionine formed (μm-mole/mg. protein)
0	2
0·08	8
0·17	24
0·33	60
0·83	250
1·66	360
3·30	420

Biological activity. The synthetic material, like the compound formed enzymically, was converted to cystathionine on incubation with extract of *Escherichia coli* strain 2/2 and a reaction mixture containing cysteine + glucose + ATP (Table 9); the further addition of homoserine or succinate did not increase the yield. The conversion was quantitative, 21·2 μmole (in terms of amino-N) giving 21·0 μmole cystathionine by microbiological assay (assuming that strain 122/33 responds to both isomers of the standard cystathionine). The elution of biological activity from Dowex-1 resin by 0·2 N-acetic acid corresponded closely with the elution of amino-N (Fig. 7). Finally, again as with the natural intermediate, biological activity disappeared rapidly on incubation at pH values above 8, the effect of increasing pH value being quantitatively similar (Fig. 5).

The effect of the cysteine concentration on cystathionine formation from the synthetic succinylhomoserine is shown in Table 9; at 0·83 mM the amount of cystathionine formed accounted for about half the cysteine added.

DISCUSSION

The evidence for and against cystathionine as an intermediate in the normal pathway of methionine synthesis by competent strains of *Escherichia coli* was discussed in the Introduction and in a previous paper (Rowbury & Woods, 1964). The already strong evidence in favour is further strengthened by the present demonstration that strains of *E. coli* contain enzymes which catalyse the formation of cystathionine from precursors indicated by the nutritional requirements of auxotrophs of the methionine series, that is, homoserine and cysteine. The work with cell-free preparations has, however, added succinate to the substrates required. We are indebted to Professor H. L. Kornberg for drawing our attention to the abstract of a communication by himself and others (Davis *et al.* 1959) relating to the properties of auxotrophs of *E. coli* which require succinate for growth and which first suggested this possibility. An auxotroph that is probably similar in nature was reported by Back & Westaway (1962). Growth in the presence of methionine represses the formation of the enzymes now shown to catalyse cystathionine synthesis (Rowbury, 1962*a*) as it does enzymes at other steps of methionine synthesis. There remains little doubt therefore that cystathionine is a normal intermediate.

The discovery that cell-free extracts of two auxotrophs, each unable to produce cystathionine, did so when mixed, indicated that the synthesis occurred in two steps and permitted study of the two reactions without the necessity of attempting the fractionation of extracts of a single strain competent to synthesize cystathionine. With one auxotroph a heat-stable intermediate compound was produced from homoserine and succinate that was converted to cystathionine by the other auxotroph in the presence of cysteine. The first step has been studied in detail and the product identified as *O*-succinylhomoserine. The second step requires further investigation; since glucose and ATP must also be present it is possible that this step is complex and that there may be a further intermediate; when reduced concentrations of cysteine were used, glucose and ATP were not required.

Formation of the intermediate *O*-succinylhomoserine by fresh bacterial extracts was increased by coenzyme A and that by an 'aged' extract by the addition of a purified preparation (from another source) of an enzyme which catalyses the synthesis of succinyl-coenzyme A. Finally, chemically prepared succinyl-coenzyme A was as effective a substrate as succinate, and, unlike the latter, did not require the additional presence of glucose and ATP. Hence it is probable that the two direct reactants are homoserine and succinyl-coenzyme A, the latter being formed in growing cultures either by the oxidative decarboxylation of α-oxoglutarate in the presence of coenzyme A (a step in the tricarboxylic acid cycle) or from free succinate, if any is formed, by the succinyl-coenzyme A synthetase which is also clearly present. With the present enzyme preparations α-oxoglutarate replaced succinate, but was less active.

The nature of the reaction, the incorporation of ^{14}C from [$^{14}C_{2,3}$]-succinate into the product and the survival in the latter of the free amino group all pointed to the intermediate being an *O*-succinyl derivative of homoserine, a conclusion supported by the identification of homoserine and succinic acid among the products after acid-treatment. The alkali-lability also indicated an *O*-derivative, since *O*-acetyl derivatives of hydroxyamino acids are similarly labile (Sakami & Toennies, 1942).

Theoretically two such derivatives are possible, *O*-succinylhomoserine and a diester in which two homoserine residues are in ester link with the two carboxylic acid groups of a single succinate residue; analysis of the acid hydrolysis products of the enzymic intermediate gave a homoserine/succinate ratio of unity, suggesting the monoester. Synthetic material was obtained from homoserine by a method in which *N*-succinylation was prevented and the presence of a free amino group in the purified product confirmed by the ninhydrin reaction; the same characteristic colour with ninhydrin as observed with the natural intermediate under certain conditions was obtained. Use of succinic anhydride as acylating agent would lead only to *O*-succinylhomoserine, but with succinyl chloride the diester might also be formed. Products obtained by both methods behaved as single (and identical) compounds on chromatography on Dowex-1 resin and it was concluded therefore that the major product in both cases was *O*-succinylhomoserine; the neutral diester would in any case have been expected to have been bound less firmly by the resin than the more acidic *O*-succinylhomoserine.

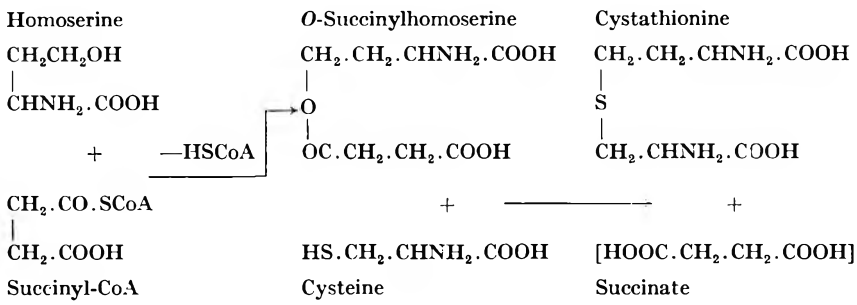

Fig. 8. Pathway of cystathionine synthesis in *Escherichia coli*.

The intermediate formed enzymically and the synthetic *O*-succinylhomoserine both gave rise to cystathionine when incubated with cysteine and cell-free extracts of the appropriate auxotroph. Their chromatographic properties on paper and Dowex-1 resin were identical, as was also their lability to strong acid and mild alkali; quantitative yields of homoserine and dibasic acid were obtained by acid hydrolysis of the synthetic compound. The product of treatment with mild alkali was not identified, but since it had no free amino group one possibility is that the succinyl residue migrates from the hydroxyl to the amino group; the release of homoserine on subsequent acid hydrolysis supports this view. The intermediate compound retains the amino group function of homoserine but has lost the hydroxyl group function; the presence of an added acidic function is indicated by the fact that it is retained by Dowex-1 resin, whereas homoserine is washed out with water. It is concluded that the natural intermediate is *O*-succinylhomoserine and the steps in cystathionine synthesis may be formulated as in Fig. 8. The name homoserine *O*-transsuccinylase is proposed for the enzyme (present in *Escherichia coli* 7/9, but not in *E. coli* 2/2) which catalyses the synthesis of *O*-succinylhomoserine.

The present work appears to provide the first case of the natural occurrence of an *O*-succinylated amino acid. *O*-acetylhomoserine is present in *Pisum sativum* (Grobbelaar & Steward, 1958) and there is a *N*-succinylation step in the synthesis of lysine by *Escherichia coli* (Gilvarg, 1960).

Cystathionine formation in *Escherichia coli* contrasts sharply with that in animal tissues, where the precursors are homocysteine and serine rather than homoserine and cysteine. The synthetase enzyme in rat liver (Binkley, 1951; Selim & Greenberg, 1959) requires no accessory substrates or cofactors other than pyridoxal phosphate, and on purification behaves as a single protein. Perhaps surprisingly, no indication of a pyridoxal phosphate requirement has so far been found in the *E. coli* system; it may, however, be tightly bound to one or more of the enzymes, as in the cystathionase of animal tissues (Matsuo & Greenberg, 1958*a*, *b*).

One of us (R.J.R.) is indebted to the Agricultural Research Council for a Studentship held during part of this work, which was aided by grants to the Department from the United States Department of Health, Education and Welfare. We are grateful to Dr L. Fowden for advice on the synthesis of *O*-succinylhomoserine.

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J. gen. Microbiol. (1964), **36**, 359–364
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Attraction Through Air Exerted by Unaggregated Cells on Aggregates of the Slime Mould *Polysphondylium violaceum*

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(*Received* 25 *March* 1964)

SUMMARY

In darkness the unitary, elongated aggregates, or grex, of *Polysphondylium violaceum* are strongly attracted through air to earlier stages of development, including pre-aggregation and feeding cells. Hence the factor responsible cannot be acrasin. This attraction is dominant over mutual grex repulsion. The significance of the response is obscure. *Dictyostelium discoideum* cells also attract *P. violaceum* grex, but do not appear to attract culminating *D. discoideum* grex.

INTRODUCTION

During the vegetative phase, cells of the collective amoeba, or cellular slime mould, *Polysphondylium violaceum*, lead a solitary existence, feeding on bacteria. When the food is exhausted, the cells enter a brief pre-aggregation phase, and then some of them found aggregation centres, which attract the others chemotactically. An aggregation centre eventually elongates perpendicularly to the agar substratum to transform itself into a cylinder with a tapered tip. This body, which behaves as an individual, is called a grex (The word 'grex' is unchanged in the plural). It constructs an axial stalk, which it proceeds to climb, at the same time extending it at the tip. An increasing length of bare stalk is uncovered as the grex advances. Periodically, small masses of cells are cut off from the rear of the grex, and these produce whorls of short side branches that are roughly perpendicular to the main stalk (Harper, 1929). The following descriptions apply only to the main stalk; the branches are too short to respond strikingly.

I previously reported (1962) that when a large number of *Polysphondylium violaceum* cells were deposited in an area about 5 mm. across in the middle of a culture plate, the grex they formed were initially more or less erect, but that soon the tips bent outwards while in mid-air, and predominantly without touching one another, so that the resultant fruiting bodies occupied a hemispherical air space, with their stalks projecting along the radii. Grex continued radially outwards even if they landed on the agar. When they were experimentally turned to point radially inwards, they soon made a U-turn to bring themselves back to their former orientation. When the cells were deposited close to the edge of the dish, most of the grex that formed nearest the glass side wall veered away from it without making contact with it; consequently a corridor next to the wall remained almost empty of fruiting bodies. Moreover, single isolated grex lying on the agar generally made a

U-turn when they approached to within a millimetre or two of the glass wall. It was thought probable that the grex were reacting by negative chemotaxis to some aerial concentration gradient resulting from their own metabolism. Bonner & Dodd (1962*b*) had come to the same conclusion after a much more thorough investigation of the reaction of four other species of slime moulds to various foreign bodies and experimental procedures.

As there was already tolerably good evidence (Samuel, 1961; Shaffer, 1962) that the cells repelled one another during the vegetative and pre-aggregation stages, and that they continued to produce the repellent material during aggregation—though its action was then usually masked by that of the attractor, acrasin—it was simplest to suppose that the grex responded to the same repellent. Against this hypothesis, I pointed out that when *Polysphondylium violaceum* was inoculated in a limited region in the middle of a thick bacterial layer, the grex that formed in this region still bent outwards, despite their being surrounded by concentric zones of all stages from feeding to aggregation, and moreover that they might even land in these zones. However, it now appears that the basis of this response is quite different from what was supposed; its nature will now be described.

METHODS

The slime mould *Polysphondylium violaceum* was grown with *Escherichia coli* on half-strength Bonner's (1947) agar (%: 0·5, glucose; 0·5, peptone; 2, agar, phosphate-buffered at pH 6·8) in Petri dishes. To exclude orientation responses to light and temperature gradients, the cultures were kept in cardboard boxes in black envelopes in a drawer lined with expanded polystyrene.

RESULTS

When *Escherichia coli* was inoculated over the whole plate, and *Polysphondylium violaceum* at one side along the edge of it, a wave of feeding amoebae slowly advanced across the agar. Behind them there was always a zone of pre-aggregation cells, and then, at most times of day, zones of aggregations and grex. The final zone contained fruiting bodies. The first fruiting bodies to be formed were sited at the edge of the dish; these were very obviously oriented away from the glass, as expected, though most of them seemed to be too nearly horizontal for them to have been escaping from a high concentration of repellent that they had themselves produced. The fruiting bodies that formed later, in the middle of the dish, pointed in the same direction as the first ones, namely, in the direction in which the feeding cells were advancing, which we shall refer to as 'forward'. Although this orientation might have resulted because there had always been grex or older fruiting bodies behind them to repel them, this explanation became highly unlikely when it was appreciated that the fruiting bodies that formed on the far side of the dish still pointed forward, and therefore *towards* the glass side-wall, even when they were closer to it than individual isolated grex had been when they had turned away from it. The stalks formed up to this point were not distributed evenly through the available air space, but had a strong tendency to bend close to the agar surface. However, when the feeding cells had advanced to the edge of the plate and had consumed all the food, the fruiting bodies that these cells then formed did veer away from the glass.

The next step was to remove all the pre-aggregation cells, aggregations, and grex present at a given moment when the feeding cells had eaten their way some distance across a plate. The first fruiting bodies to be built ahead of the empty swath still leaned forward very markedly, even though there were no grex behind them, or even any close fruiting bodies, that could have repelled them. There was therefore a strong suggestion that the grex were being attracted forwards. This was shown yet more convincingly by inoculating the slime mould at one end of a narrow bacterial streak. Had the orientation of the grex been dominated by their mutual repulsion, their stalks should have projected in planes perpendicular to the streak axis. In fact, the great majority of them pointed straight forwards along it—those formed in the mid-line of the streak pointing forwards soon after they rose from the agar, those nearer the edge first growing out slightly to the side and then turning forwards. In contrast, when the slime mould was initially inoculated along the whole length of the streak, the resultant stalks were distributed in planes transverse to the streak. It might therefore be concluded that when all the amoebae present were at the same stage of development, the orientation of the grex was greatly influenced by mutual repulsion; but that when grex developed close to bacteria and earlier stages of development, they were strongly attracted towards them, and that this attraction was dominant over repulsion.

This raised the question as to whether it was the bacteria or the amoebae that were attractive, and if the amoebae, at which stages. A strong hint as to the answer was given by the observation, already reported, that often the largest grex landed on the agar among the feeding and pre-aggregation cells. The reason why only the largest grex could make an actual landing was simply that only they made stalks long enough for them to arch over the intervening zone of aggregation. This behaviour had not previously seemed remarkable, for the following reasons. Newly formed grex of *Dictyostelium discoideum* usually migrate for some time over the agar surface, without constructing a stalk, before they become erect again and turn into stalked fruiting bodies (Raper, 1935). (This re-erection itself poses a problem in orientation, but we are not concerned with it here.) It has long been known that the stalked grex of other species, including *Polysphondylium violaceum*, may pass through a phase believed to be comparable with migration, in which they crawl on their sides over the agar surface (Raper, 1941). Also, since mutual repulsion may make the peripheral members of a stand of grex travel outwards at a rather oblique angle to the agar surface even during an aerial phase, and since long stalks when nearly horizontal are mechanically rather unstable, it might be expected that grex at the end of such stalks would tend to land on the agar. However, it was found during the present work that when the culture plate was incubated upside-down, the large grex still managed to land on the agar among the feeding and pre-aggregation cells. Clearly therefore the grex were not reacting to gravity but were being led towards these cells. The grex often buried themselves so deeply in the thick layer of feeding slime-mould cells that they became virtually invisible, except for their cellulose stalks and extreme tips. Nevertheless, their development continued.

The reactions of the grex were examined by cutting out a rectangular block a few centimetres square from agar of standard thickness and bearing cells of any desired stage of development, and placing it alongside a block carrying a row of large aggregation centres. To prevent cells wandering from one block to the other, a strip of

polystyrene film was inserted between them of such a height that it projected just above the agar surface. In this way, it was clearly shown that grex were strongly attracted to feeding cells as well as to pre-aggregation cells. Young aggregations seemed to be rather less attractive. However, although the different test blocks were taken from similar cultures, and therefore bore cells at approximately the same population density, no accurate comparison between these stages was attempted. The main object of these experiments was to determine whether or not the grex were attracted to amoebae that had not begun to aggregate. Plate 1, fig. 1, shows a simpler version of this demonstration: aggregating centres were merely deposited in a single line next to the edge of a band of feeding amoebae several centimetres wide.

A few similar experiments were performed with *Dictyostelium discoideum*. Carrying the aggregation centres through the air to deposit them in a line subjected them to sufficient desiccation to eliminate the migration phase, and the grex proceeded to culminate where they were deposited. The resultant fruiting bodies did not incline towards the adjacent band of feeding cells; on the contrary, in general they leant away from it (Pl. 1, fig. 2). But *D. discoideum* cells did attract grex of *Polysphondylium violaceum*.

The possibility that the bacteria used (*Escherichia coli*) were primarily responsible for the attraction had still not been completely excluded, although it seemed most unlikely, as there were virtually no uningested bacteria left between the pre-aggregation cells. Yet these cells did still contain some bacteria, and presumably smelt of the *E. coli* they had digested. Test blocks were therefore cut from plates inoculated solely with *E. coli*. These did not strikingly attract grex of *Polysphondylium violaceum*, although the experiments were not precise enough to show whether or not the bacteria were very weakly attractive.

On the basis of the above results, the following more striking method of showing the attraction was devised. A circular area about 1 cm. in diameter was inoculated with *Escherichia coli*, and *Polysphondylium violaceum* spores were added all round the perimeter of this area. The amoebae ate their way inwards till they met in the middle. By the time grex had developed at the periphery, the middle was packed with amoebae that had completely or almost completely consumed the bacteria there, as shown by the increase in transparency. In these conditions, the grex oriented radially inwards (Pl. 1, fig. 3), and the end result was a tangle of fruiting bodies lying on the agar. In contrast, when the initial diameter of the inoculum was sufficiently small for there to be no unaggregated cells in the middle when the grex sprang up at the edge, these formed the usual graceful fruiting bodies, oriented radially outwards.

DISCUSSION

In *Polysphondylium violaceum*, the onset of aggregation is abrupt (Shaffer, 1961), and before that the amoebae do not release acrasin, at least in detectable amounts. It is therefore clear that grex are attracted to unaggregated cells by something other than acrasin. Although it seems probable that the effective agent in attraction is a gas or a volatile metabolite, no attempt has been made to demonstrate this directly. Bonner & Dodd (1962*b*) conducted many experiments that tended to show that this was true of the factor by which the grex repelled one another, but they pointed out that conclusive evidence was still lacking. Since then, I have observed aerial grex

responses to such a variety of distant materials that it has become still more improbable that there could be a purely physical explanation for all of them.

Bonner & Dodd (1962*b*) showed that grex were strongly attracted towards activated charcoal, and they interpreted this to mean that the charcoal adsorbed the repellent, and hence that the grex responded to it by negative chemotaxis. So perhaps unaggregated cells are attractive because they efficiently absorb a repellent made by the grex. Yet this seems unlikely, for in the last experiment described, the grex crowded radially inwards to form almost a solid mass, instead of turning towards the rest of the culture plate which was completely unoccupied. In any case, if the individual cells repel one another, as some evidence suggests they do (Samuel, 1961; Shaffer, 1962), there must be at least two factors: either the grex make a different repellent; or they make the same repellent as the separate cells, and these cells must also make an attractor.

Bonner & Hoffman (1963) believe that one and the same 'spacing substance' is responsible for mutual grex repulsion and for preventing too many aggregations from forming in a given area (Shaffer, 1962; Bonner & Dodd, 1962*a*, *b*). They found that the same factor was produced by all the species they tested, including *Polysphondylium violaceum* and *Dictyostelium discoideum*, but that at the aggregation stage *D. discoideum* was insensitive to it. By the grex stage, *D. discoideum* had become sensitive to it; that is to say, culminating Dictyostelium grex repelled one another and were also repelled by grex of Polysphondylium. In the present work it has been found that cells of both species can attract grex of *P. violaceum*. However, culminating *D. discoideum* grex appear not to be attracted. Perhaps they are attracted covertly, but in the conditions used, repulsion is dominant. Certainly when attraction and repulsion occur simultaneously, the balance may be subtle, as I have elsewhere (1962) discussed with reference to aggregation.

Has grex attraction any adaptive value? Bonner & Dodd (1962*b*) and Bonner & Hoffman (1963) suggested that it is advantageous for the organism to space out its fruiting bodies, as well as its aggregations. The repulsion between individual feeding and pre-aggregation cells may also be interpreted as an adaptation to finding food. At first sight, this argument accords perfectly with the newly discovered phenomenon: the grex are attracted towards feeding cells. But this extension of the argument does not survive examination. The grex are not attracted strongly to food itself but to feeding cells, and to cells that have consumed all the available food. A more serious objection is that even if this response does sometimes lead a grex to food, it could be considered adaptive only if the grex could take advantage of the food it had found. In fact, although a very small proportion of the peripheral grex cells do return to the vegetative stage, a grex as a whole continues to develop into a fruiting body even when it has buried itself in the layer of feeding cells or of bacteria. Indeed, even if aggregated *Polysphondylium violaceum* cells are mechanically disaggregated in the presence of bacteria, most of them immediately reaggregate (Shaffer, 1961).

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EXPLANATION OF PLATE

Fig. 1. Aggregation centres of Polysphondylium violaceum were deposited on the bare agar in a single row just beyond the edge of a broad band of homologous feeding cells. The resultant grex have all oriented in the dark towards the feeding cells, with the exception of the two smallest grex, one of which has arisen directly opposite a large one and may have been repelled by it.

Fig. 2. An experiment identical with that shown in fig. 1, except that the species is Dictyostelium discoideum. The bare agar (above) is covered with tracks produced by a very small number of separate cells. The fruiting bodies are more erect than those of the grex seen in fig. 1, but they lean predominantly away from the feeding cells.

Fig. 3. The great majority of P. violaceum grex that have formed around the periphery of a circular area of feeding cells have oriented almost radially inwards and are nearly horizontal.

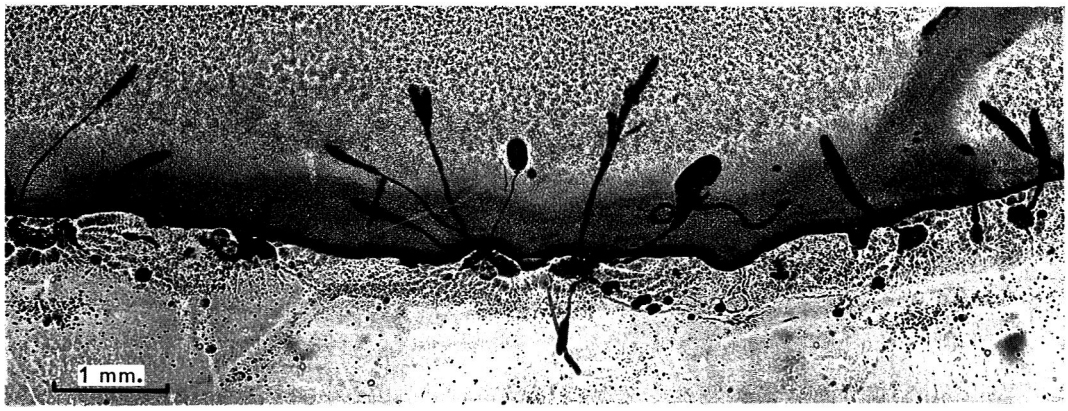

Fig. 1

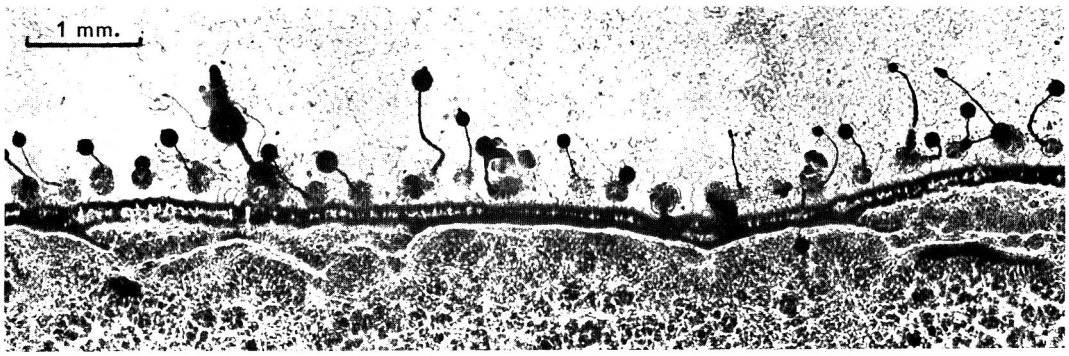

Fig. 2

Fig. 3

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J. gen. Microbiol. (1964), **36**, 365–384
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Transfer of the Colicin I Factor in *Escherichia coli* K12 and its Interaction with the *F* Fertility Factor

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(*Received* 26 *March* 1964)

SUMMARY

A series of *Escherichia coli* K12 strains of various mating types was made resistant to colicin I (*colI-r*) and colicinogenic for this character (*colI*$^+$). In the absence of the *F* sex factor, the number of 'competent donors' of *colI* in broth cultures of colicinogenic strains was about 1 %. Transfer of *colI* to non-colicinogenic strains reached about 7–10 % within 2 hr of mixing, and between 50 and 100 % after incubation overnight. Cells newly infected with *colI* transfer more efficiently, and can infect over 90 % of a recipient strain with *colI* within 1 hr of mixing (high-frequency colicinogeny transfer, HFC). When the *F* factor was present in the colicinogenic donor strain of a mixture, *colI* transfer was reduced; when *F* was present in the non-colicinogenic recipient, *colI* transfer was increased. The colicin *I* factor appeared to be transferred by a series of Hfr strains as an extra-chromosomal element.

INTRODUCTION

Many strains of Enterobacteriaceae produce the protein-like antibiotics known as colicins, which have been subdivided by Frédéricq (1957) into a number of types. The production of each colicin is presumed to be controlled by a stable genetic determinant called a colicin factor (*col* factor; thus *colI* determines the production of colicin I), cells harbouring such factors being termed colicinogenic. Colicinogeny shows many similarities with lysogeny (the ability to produce a temperate bacteriophage; see Frédéricq, 1958, 1963*a*, *b*). Probably the most striking is the demonstration by Ozeki, Stocker & de Margerie (1959) that colicin production is due to a lethal synthesis. Colicinogenic bacteria carry a potentiality to synthesize colicin but this is normally expressed only by a small random proportion of cells in the population, which are killed in the process.

Certain strains can transfer their colicinogenic property to non-colicinogenic strains during mixed cultivation (Frédéricq, 1954). This transfer requires cell-to-cell contact, and takes place in the absence of any observable transfer of chromosomal genes. The colicin factors (whose transfer is inferred under these conditions) were thus suggested to exist as extra-chromosomal or cytoplasmic elements (Frédéricq & Betz-Bareau, 1953).

The transfer of colicin factors responsible for the maintenance of several distinct colicins has been investigated in *Salmonella typhimurium* (Ozeki, Stocker & Smith, 1962; Smith & Stocker, 1962; Stocker, Smith & Ozeki, 1963; Smith, Ozeki & Stocker, 1963). The factors *colI* and *colB* were found to be transferred readily, whereas *colK*, *colE 1* and *colE 2* were transferred very infrequently or not at all. The

infective factors *colI* and *colB* were also capable of mediating the transfer of normally non-infective factors such as *colE1* and *colE2*. Moreover, cells to which the *colI* factor had been recently transferred were found to have a greatly enhanced efficiency of transfer ('high-frequency colicinogeny transfer', HFC) giving rise to an 'epidemic spread' of *colI* within newly infected cultures (Stocker *et al.* 1963).

In Escherichia coli K12, *colI* is also transferred, in the absence of the *F* sex factor (Clowes, 1961). The present paper reports experiments on the kinetics of this transfer, on the effect of the presence of the *F* factor, either in the autonomous (F^+) or in the integrated (Hfr) state, on this process.

METHODS

Bacterial strains used. The strains are specified in Table 1. The 'origines' and directions of transfer of the Hfr strains are shown in Fig. 1. The *colI* factor in all strains originates from *Shigella sonnei* P9, transmitted to *Salmonella typhimurium* LT2 (Ozeki *et al.* 1962), and is thus identical in origin with that used in previous investigations (Ozeki & Howarth, 1961; Clowes, 1961; Ozeki *et al.* 1962; Smith & Stocker, 1962; Stocker *et al.* 1963; Smith *et al.* 1963).

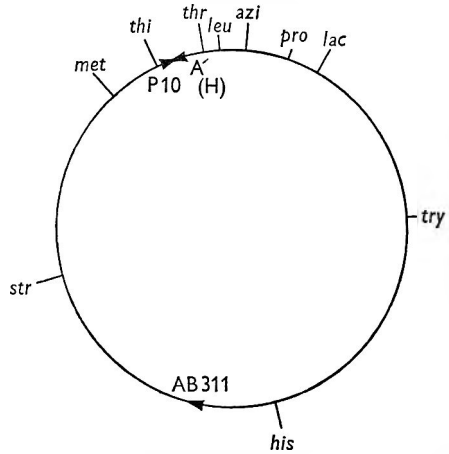

Fig. 1. The circular linkage group of Escherichia coli K12. The relative positions of the genetic markers are drawn on the outside of the circle. The arrows on the circle represent the leading extremity and direction of transfer of the chromosome in the various Hfr strains denoted on the inside of the circle.

Media. Nutrient broth was prepared from Oxoid No. 2 broth powder at 25 g/l. adjusted to pH 7·2; nutrient agar was made by the incorporation of Davis agar powder at 12 g/l. Minimal agar was, as described by Tatum & Lederberg (1947) with the omission of asparagine, solidified with Davis agar at 15 g./l. and supplemented with D-glucose at 2 g./l. Amino acid supplements were added at 20 mg./l. and vitamin B1 (thiamin) at 5 mg./l. Soft agar used in overlays was made up as Difco agar at 6 g./l. without nutrients or salts and held molten at 45°. Streptomycin was added at 200 mg./l., and azide as sodium azide at M/500 in nutrient agar or M/1600 in minimal agar. Eosin methylene blue (EMB) and tetrazolium media were prepared according to Lederberg (1950*b*), supplemented with either lactose or maltose to score for sugar fermentation.

Table 1. *Bacterial strains*

Strain no.	Genotype	Origin
(*a*) Strains derived from *Escherichia coli* K12, 58–161 (*met*) Tatum & Lederberg (1947)		
501	F+	Original strain, 58–161 (Hayes, 1952)
518	F−	501 by loss of *F* (Hayes, 1953)
519	F− *colI*+	518 infected with *colI* from 902
524	F− *colI*+ *str-r*	519 made resistant to streptomycin
535	F+ *colI-r*	501 resistant to colicin I from 902
536	F+ *colI*+	501 infected with *colI* from 902
544	F+ *colI-r str-r*	535 made resistant to streptomycin
547	F− *colI-r*	518 resistant to colicin I of 902
550	F− *colI-r str-r*	547 made resistant to streptomycin
559	F− *colI*+ *colI-r*	547 infected with *colI* of 519
(*b*) Strains derived from *E. coli* K12, Hfr Hayes (*met str-r azi-r T 1-r*)		
715	Hfr H*	Original strain (Hayes, 1953)
730	Hfr H *colI-r*	715 made resistant to colicin I of 519
732	Hfr H prototrophic *mal*− *gal*− *ara*− *str-s*	*str-s* Hfr recombinant. Otherwise known as *A'Hfr* (Hayes, personal communication)
733	Hfr H prototrophic *colI*+ *mal*− *gal*− *ara*− *str-s*	732 infected with *colI* of 519
735	Hfr H prototrophic *colI-r mal*− *gal*− *ara*− *str-s*	732 made resistant to colicin I of 559
(*c*) Strains derived from *E. coli* K12 HfrAB311 (*thr leu str-r T 6-s*)		
741	HfrAB311*	Original strain (Taylor & Adelberg, 1960)
763	HfrAB311 *colI*+	741 infected with *colI* from 519
(*d*) Strains derived from *E. coli* K12, HfrP10 (*thr leu thi mal*−*lac*− *gal*− *T 1-r T6-s*)		
749	HfrP10*	Original strain Hfr P10, equivalent to Hfr(J)4. (Jacob & Wollman, 1958;
764	HfrP10 *colI*+	749 infected with *colI* of 519
(*e*) Strains derived from *E. coli* K12, J6-2 (*pro his try str-s*)		
710	F+	Original strain (Clowes & Rowley, 1954)
711	F−	710 cured by acridine orange†
712	F− *str-r*	711 made resistant to streptomycin
707	F− *str-r colI-r*	712 made resistant to colicin I from 902
765	F− *str-r colI-r T6r*	707 made resistant to coliphage T6
(*f*) Strains derived from *E. coli* K12, W677 (*thr leu thi mal*− *lac*− *gal*− *xyl*− *mtl*− *ara*−) Lederberg, 1950*a*)		
27	F+ *str-r colI-r*	28 infected with *F* from 501
28	F− *str-r colI-r*	30 made resistant to streptomycin, and to colicin I from 902
30	F−	Original strain W677 (Hayes, 1952)
46	F− *colI*+	30 infected with *colI* from 519
(*g*) Strains other than *E. coli*		
902	*Salmonella typhimurium* LT2 *cys D*-36 *colI*+	LT2 *cys D*-36 infected with *colI* from *Shigella sonnei* P9 (Ozeki *et al.* 1962).

Growth requirements: *met*, methionine: *thr*, threonine: *leu*, leucine: *thi*, thiamin: *pro*, proline, *his*, histidine: *try*, tryptophan.

Resistance markers; *str-r*, resistant to streptomycin: 200 μg./ml.: *azi-r*, resistant to sodium azide: *colI-r*, resistant to colicin I: *T*1-*r*, *T*6-*r*, resistant to the coliphages T1, T6: *colI*+ = colicinogenic for colicin I.

Sugar fermentations; *mal*, maltose: *lac*, lactose: *gal*, galactose: *ara*, arabinose.

* See Fig. 1 for 'origine' and orientation of transfer of these strains.

† Cured by method of Hirota (1960).

Techniques

Colicinogeny was tested by one of several methods. In the stab method (Frédéricq, 1957), colonies (including control col^+) were inoculated by straight wire into nutrient agar. After overnight incubation, the plate was sterilized by exposure to chloroform vapour, and then overlayed with 2·5 ml. soft agar, seeded with 0·1 ml. of an overnight broth culture of a strain sensitive to the colicin (indicator strain). After further overnight incubation, the indicator cells grow as a confluent lawn except around the site of inoculation of a col^+ colony, where a zone of inhibition is observed. In the triple overlay (or sandwich plate) technique, the culture to be tested was diluted to give 10^3 to 10^4 cells /ml., 0·1 ml. of this dilution being plated in 2·5 ml. of soft agar overlay. When this overlay had set, a further 2·5 ml. of unseeded soft agar was plated, to prevent emergence of any of the seeded bacteria as surface colonies. After overnight incubation, the identification of those colonies appearing within the agar overlay which are col^+ was achieved by a third overlay containing indicator bacteria, followed by a second period of incubation. A colony at the centre of the circular inhibition zone may then be 'fished' from the soft agar, and propagated as a stable col^+ strain.

Free colicin was assayed to give a measure of the colicin titre in cultures of known colicinogenic strains. Drops from a series of doubling dilutions of a culture filtrate or supernatant fluid from centrifugation were spotted on a nutrient agar plate, previously overlayed with an indicator strain. When the col^+ strain was *str-s*, a *str-r* indicator was used on streptomycin nutrient agar. Alternatively the culture was freed from viable bacteria by incubation for about 30 min. at 37° after the addition of a few drops of chloroform.

Colicin resistant (col-r) strains were produced from a parental *col-s* strain by its use as an indicator overlay to a chloroformed col^+ stab plate. After incubation for 48 hr, several isolated colonies usually appeared within each inhibition zone. Cultures from these colonies no longer produced inhibition zones when used as indicators, and were now colicin-resistant.

Isolation of colicinogenic strains was achieved by inoculating 0·1 ml. of an overnight broth culture of the strain to be made colicinogenic (recipient) into 5 ml. nutrient broth, together with 1 loopful of a colicinogenic strain (donor), to achieve a ratio of donor to recipient of about 1:20. After overnight incubation, the recipient was re-isolated by the use of either a differentiating (EMB or tetrazolium) medium, or a selective medium (streptomycin or azide nutrient medium, or appropriately supplemented minimal medium). Individual colonies were then tested for the acquisition of colicinogeny by either the stab or sandwich plate techniques.

Kinetics of transfer of colicinogeny at low frequencies (LFC; see Ozeki *et al.* 1962). Overnight cultures of a col^+ *str-s* donor strain, and a non-colicinogenic (col^-) recipient strain (previously made resistant to both colicin I and streptomycin) were each diluted 1/20 into broth and re-incubated for 90 min. to produce logarithmic (log) cultures at about 10^8 organisms/ml. Equal volumes were then mixed and re-incubated without shaking or aeration. At various times, samples were removed, gently but rapidly diluted (at early times 10^{-4}, at later times 10^{-5}) and 0·1 ml. volumes plated in soft agar on streptomycin nutrient agar plates and further overlayed with unseeded soft agar. After overnight incubation only the *str-r* recipient strain

produced colonies within the agar. Overlay with a *col-s str-r* indicator strain allowed the colonies which have acquired colicinogeny to be easily recognized by their surrounding inhibition zones. Between 300 and 1000 colonies were tested for the acquisition of *colI* at each time of sampling.

Kinetics of high frequency colicinogeny transfer (*HFC*) was followed from a HFC '*donor mixture*' to a recipient, by the method of Stocker *et al.* (1963). Five ml. nutrient broth were inoculated with about 5×10^4 cells from a *colI*$^+$ *str-s* strain (donor) and about 10^6 cells of a *col*$^-$ *colI-r str-s* (intermediate) strain. After overnight incubation, the mixture was diluted 1/20 in fresh broth and re-incubated a further 90 min. with aeration to about 10^8 cells/ml. Almost 100 % of the intermediate cells in such an HFC 'donor mixture' were *colI*$^+$ and since a high proportion of them had recently acquired colicinogeny, they showed a high efficiency of transfer when mixed with the recipient, a *colI*$^-$ *colI-r str-r* strain. Log. phase cells of an HFC donor mixture and a recipient, both at 10^8 organisms/ml. were mixed in broth and the transfer of *colI* to the recipient was followed by plating samples at intervals on streptomycin agar and assaying for *colI*$^+$ colonies.

The number of '*competent donors*' in a *col*$^+$ population was determined according to Ozeki *et al.* (1962). 0·2 ml. samples of a culture of a *col*$^-$ *str-r* strain were added to a series of tubes containing 2 ml. pre-warmed broth. A culture of the *colI*$^+$ *str-s* strain to be assayed (at about 5×10^8 organisms/ml.) was diluted serially tenfold, and from dilutions containing about 50,000, 5000 and 500 cells/ml., 0·1 ml. samples were added to each of 15 (or 25) tubes containing the *col*$^-$ inoculum. The tubes were incubated 15 min., streptomycin (to 200 μg./ml.) was added to each, and incubation continued overnight; 0·1 ml. of a 10^{-5} dilution from each tube was then plated in soft agar and tested for the presence of *col*$^+$ colonies in the usual way.

Cell to cell contact was observed in mixture of two strains, one of which had been stained with tetrazolium. An efficient method of staining is due to Drs E. & G. Kellenberger (personal communication). To an actively growing culture at a density of about 10^8 organisms/ml., vigorously aerated in broth (pH 7–7·5), a solution of tetrazolium (2,3,5-triphenyltetrazolium chloride) is added to a final concentration of 0·1 % and aeration immediately stopped. After further incubation for 5 min. the culture turns bright red, and the great majority of cells can be seen under the microscope to show 1 or 2 sites ('formazan granules') which are intensely stained. Specific pairing or clumping between cells may thus be observed in mixtures in which only one of the strains is so labelled.

F$^+$ *testing* was achieved by a modified screening method, making use of the male specific phage μ (Dettori, Maccacaro & Piccinin, 1961). Inocula from each of 50 to 100 presumptive F$^+$ colonies were taken into tubes containing 2 ml. broth, which were then incubated overnight. One loopful from each tube was spread over an area of about 2 cm. diameter on a nutrient agar plate (about 18 spread areas/plate, including F$^+$ and F$^-$ controls). By using a sterile glass syringe with a fine needle, one drop (0·01 ml.) of a 10^{10} particles/ml. suspension of μ phage was then placed at the centre of each spread area. After incubation for 6–8 hr, only the *F* infected strains showed a region of phage lysis within the surrounding area of bacterial growth.

F$^+$ *and Hfr mediated recombination.* Logarithmic phase cultures of donor and recipient strains at about 10^8 cells/ml. were mixed in equal volumes and incubated together for 2 hr, and 0·1 ml. samples were then plated (after suitable dilution

from Hfr crosses) on appropriately supplemented minimal agar to yield several hundred recombinant colonies after incubation for 48 hr.

Interrupted mating in Hfr crosses was achieved by removal of 0·1 ml. samples from donor + recipient mixtures during the 2 hr incubation period into 1 ml. of 10^{10} particles T6 phage/ml. Incubation was continued for a further 15 min. when the samples were vigorously agitated for 1 min. at top speed in a 'Microid' flask shaker (Griffin & George, London), rapidly diluted, and plated on selective minimal media for recombinants as above.

RESULTS

Isolation and characteristics of K12 strains colicinogenic for colicin I

In general, the *colI* factor was readily transferred to a variety of K12 strains during mixed incubation of *colI*$^+$ and non-colicinogenic (*col*$^-$) strains. The *col*$^-$ strain was not killed by free colicin I in the culture, which permitted the isolation of drug-sensitive (*str-s* or *azi-s*) *colI*$^+$ strains on differentiating medium. After overnight mixed cultivation, usually between 50 and 100% of the surviving *col*$^-$ colonies acquired *colI*, irrespective of whether Salmonella or Escherichia strains were used as colicinogenic donors.

From early experiments, the sizes of the inhibition zones produced by *colI*$^+$ strains of *Escherichia coli* (3 mm. from an 18 hr 'stab', or 2 mm. from a 24 hr deep-agar colony) were found to be smaller than those produced by Salmonella under similar conditions (10 and 6 mm., respectively). This difference was ascribed to the adsorption of colicin by the non-producing cells of the colonies of *E. coli*. Thus, when the *E. coli* strain made *colI*$^+$ had previously been made resistant to the colicin (*colI-r*) its inhibition zones were larger, and were in fact similar in size to those produced by Salmonella which has been shown to be naturally colicin-resistant (Ozeki *et al.* 1962). That the zone sizes were due entirely to the presence or absence of the colicin-resistance marker, and not to an alteration of the colicin I factor, was shown by the fact that transfer of *colI* to a colicin-sensitive strain from either a resistant (*colI*$^+$ *colI-r*) or a sensitive (*colI*$^+$ *colI-s*) donor produced a strain (*colI*$^+$ *colI-s*) which gave small inhibition zones, whereas infection of a colicin-resistant strain from either donor gave a *colI*$^+$ *colI-r* strain which produced the larger inhibition zones. Further experiments showed that the amount of free colicin in cell-free filtrates of overnight *colI*$^+$ *colI-r* cultures allowed a sixteenfold dilution whilst still producing inhibition when drops were plated on a lawn of *col-s* indicator cells, whereas a parallel *colI*$^+$ *colI-s* culture filtrate produced only very slight inhibition when similarly plated without dilution, and did not do so after even two-fold dilution. The majority of cells in a culture of a colicinogenic strain do not produce colicin (Ozeki *et al.* 1959), and it appeared likely that these cells in a *colI*$^+$ *colI-s* culture could absorb the liberated colicin, whereas cells of a *colI*$^+$ *colI-r* culture have lost this ability with their mutation to colicin resistance. This conclusion is supported by the finding that the end-point dilution of inhibition from 1 + 1 mixtures of overnight cultures of *colI*$^+$ *colI-r* and *colI*$^+$ *colI-s* strains was decreased to that of the *colI*$^+$ *colI-s* cells after mixing for 3 hr. at 37°.

Number of 'competent donors' of colI in K12F$^-$ strains

The proportion of colicinogenic cells which are able at any one time to transmit their *col* factor (competent donors) was determined by Ozeki *et al.* (1962) by measur-

ing the number of *colI*$^+$ cells able to initiate an epidemic spread of colicinogeny within a large number of non-colicinogenic cells. A similar estimation was made for *Escherichia coli colI*$^+$ strains (Table 2). After overnight incubation of serial inocula of *colI*$^+$ *str-s* cells with a large excess of *colI*$^-$ *str-r* cells, samples were plated from the tubes and assayed for the presence of *colI*$^+$ *str-r* cells, and were found to be made up almost entirely of either *colI*$^+$ (>75 %) or *col*$^-$ ($<0{\cdot}01$ % *colI*$^+$) cells. The proportion of tubes for each size of donor inoculum which did not show any *colI*$^+$ cells

Table 2. *'Competent' donors in broth cultures of Escherchia coli K12 colI*$^+$ *F*$^-$ *strains*

Samples (0·2 ml.) of serial tenfold broth dilutions of either log phase (log) or overnight (O/N) cultures of a *colI*$^+$ *str-s* donor strain (519, 559 or 46) were mixed with 0·2 ml. of overnight cultures (3×10^8 cells/ml.) of a non-colicinogenic *colI-r str-r* recipient strain (550), 15 (or 25) tubes being mixed at each broth dilution. After 15 min. incubation, 2 ml. of streptomycin nutrient broth was added to each tube and incubation continued overnight. 0·1 ml. of a 10^{-5} dilution from each tube was then plated on streptomycin agar and the resulting colonies tested for acquisition of *colI*. The mean number of competent donors/tube was calculated from the proportion of tubes in which there were no *colI*$^+$ bacteria after overnight incubation, using this value as the zero term of the Poisson equation, e^{-m}, where m = mean number of competent donors/tube.

Strain no.	No. of *colI*$^+$ donor bacteria added per tube	Nos. of tubes giving negative or positive test for presence of *colI*$^+$ recipient bacteria		Mean nos. of 'competent donors' per tube	Average nos. of *colI*$^+$ donor bacteria per 'competent donor'
		−	+		
519 (log)	9·2	14	1	0·07	132
	92	2	13	2·03	45
	921	0	15	>2·71	<364
519 (O/N)	2·9	24	1	0·04	72
	28·6	20	5	0·22	130
	286	1	19	2·95	95
559 (O/N)	2·9	25	0	<0·05	>59
	29·2	19	6	0·27	108
	292	0	20	>3·0	<98
46 (O/N)	2·6	24	1	0·04	64
	25·6	21	3	0·13	197
	256	4	14	1·5	170

was used as the zero term of the Poisson equation to estimate the average numbers of competent donors added to each tube for this donor inoculum. From Table 2 it can be seen that from either log-phase or overnight cultures of both 58–161 and w677 strains, the proportion of competent donor cells was probably between 1 in 50 and 1 in 200.

Kinetics of low frequency colicin transfer (LFC) of colI in the absence of the F factor

In preliminary experiments, the transfer of *colI* from a *colI*$^-$ *str-s* to a *col*$^-$ *str-r* strain after mixed growth for 18 hr varied between 20 and 100 %. These fluctuations were evidently due to variations in population density and input ratios of the two strains as shown in Fig. 2. Transfer of *colI* began within 2 hr of mixing and was most rapid at the highest donor to recipient ratio (curve A). With ratios of 2:1 or less (curves, B, C, D), transfer, although initially lower, increased after the first few

hours, the increase now being most marked in mixtures of the lowest donor : recipient ratio curves (C and D), where the value reached was 70–90 % after incubation for 18 hr. With a 1 : 1 input at about 10^8 cells/ml., transfer was about 7–10 % after the first 4 hr, attaining 80 % after 18 hr. This input ratio and cell concentration was used in all succeeding experiments.

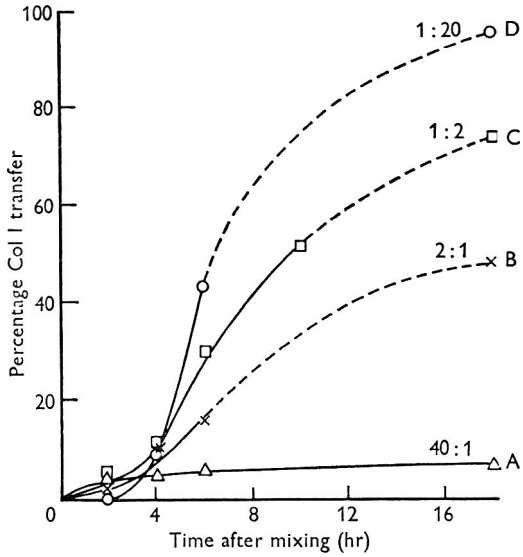

Fig. 2

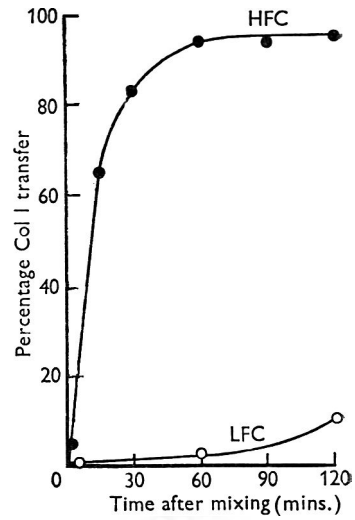

Fig. 3

Fig. 2. The effect of donor to recipient ratios on *colI* transfer under LFC. Log. phase donor strain 519 (F^- *colI*$^+$ *str-s*) and recipient 550 (F^- *colI-r str-r*) were diluted and mixed at the following population densities/ml.

Strain 519	Strain 550	Ratio	Curve	
$1{\cdot}2 \times 10^8$	$2{\cdot}7 \times 10^6$	40 : 1	A	△——△
$1{\cdot}2 \times 10^8$	$6{\cdot}7 \times 10^7$	2 : 1	B	×——×
$3{\cdot}2 \times 10^7$	$6{\cdot}7 \times 10^7$	1 : 2	C	□——□
$3{\cdot}4 \times 10^6$	$6{\cdot}7 \times 10^7$	1 : 20	D	○——○

The mixtures of organisms were incubated and at various times samples were removed, diluted and plated on streptomycin agar. After incubation the resulting recipient clones were tested for colicinogeny by overlay with a colicin-sensitive indicator.

Fig. 3. Low and high frequency *colI* transfer. Lower curve (○——○)LFC. Log. phase cultures of donor 519 (*colI*$^+$ *str-s*) and recipient 550 (*colI*$^-$ *str-r*) at about 10^8 organisms/ml. each were mixed in equal volumes and incubated. At various times samples were plated on streptomycin agar and the resulting colonies tested for *colI*$^+$.

Upper curve (●——●)HFC. Five ml. nutrient broth were inoculated with 5×10^4 cells of 519 donor and 10^6 cells of an intermediate (*colI*$^-$ *str-s*) strain 547. After overnight incubation the mixture was diluted 1 to 20 in 5 ml. fresh broth and incubated with aeration for 90 min. (to about 10^8 cells/ml.). This HFC 'donor mixture' was mixed with a log. phase culture (at 10^8 cells/ml.) of the recipient strain 550 and the mixture incubated. At various times samples were withdrawn and the recipient selected and tested for *colI*$^+$ as above.

The transfer of *colI* in cultures of *Escherichia coli* thus appears to be a result of two distinct processes, a low initial rate of transfer and a rapid subsequent rate of transfer. In this way it resembles the 'epidemic spread' of *colI* in *Salmonella typhimurium* described by Stocker *et al.* (1963) which was found to be due to the greatly enhanced ability of cells newly infected with *colI* to transfer this factor.

On this interpretation, the initial transfer shown in Fig. 2 would be due to a high donor:recipient ratio, producing an initially high rate of transfer. This is followed by transfer from newly infected recipient cells (recipient to recipient transfer) which is more extensive in cultures of low donor:recipient input, where the probability of recipient to recipient contacts is increased. The occurrence of high frequency colicinogeny transfer (HFC) in *E. coli*, analogous to that found in *S. typhimurium* by Stocker *et al.* (1963) was therefore investigated.

Kinetics of high frequency colicinogeny transfer (HFC) of colI in the absence of the F factor

Equal volumes of an 'HFC donor mixture' prepared according to Stocker *et al.* (1963) and a log phase culture of a non-colicinogenic *colI-r str-s* recipient strain, both at about 10^8 cells/ml. were mixed and incubated without aeration. At various times, diluted samples were plated in soft agar overlays on streptomycin agar and the colonies formed after incubation for 24 hr tested for the acquisition of *colI*. The kinetics of *colI* transfer are shown in Fig. 3, together with normal (LFC) transfer for comparison. Transfer from the HFC donor mixture occurred at a very rapid rate, about 60–70 % of the recipient cells having acquired *colI* within the first 15 min. after mixing, and more than 90 % after 1 hr.

The time taken for transfer of the colI factor by HFC cultures was estimated by mixing an HFC donor mixture and a recipient strain, diluting after 1 min. and then rapidly agitating in a 'Microid' shaker to separate cell pairs; under these conditions 30–40 % of the recipient cells acquired colicinogeny, and within 5 min. after dilution, the great majority (> 90 %) of the recipient cells were colicinogenic.

The duration of high infectivity after acquisition of colI was determined from an HFC mixture prepared in the usual way, but in which incubation was continued beyond the normal 90 min. period, with dilutions into fresh warm broth at 30 min. intervals to maintain the concentration of organisms approximately constant. At various times, samples were removed, assayed for viable count, and then mixed with about 10^8 cells of a recipient culture. After 10 min. to permit cell contact, each mixture was gently diluted 10^{-5} into buffer and after incubation for 1 hr was plated on streptomycin agar. The transfer of *colI*$^+$ to the recipient was assayed in the usual manner. Table 3 shows that an increased transfer efficiency persisted for 2–3 hr although during this period a progressive decay in the degree of transfer took place.

The effect of the F sex factor on colI transfer under LFC conditions

The effect of *F* on *colI* transfer under LFC conditions was investigated by using strains in which the *F* factor was either autonomous (F^+) or integrated (Hfr). In the classical fertility cross 58–161 $F^+ \times$ w677 F^- where *F* is autonomous (Hayes, 1953), *F* transfer is high and reaches close to 100 % in 1 or 2 hr. In contrast, *F* transfer between the strains 58–161 $F^+ \times$ 58–161 F^- is much less efficient and attains a value of only 10–20 % in the first 5 hr and usually no more than 30 % after overnight mixed cultivation (similarly, low efficiencies of *F* transfer are also found in the mixtures w677 $F^+ \times$ 58–161 F^- and w677 $F^+ \times$ w677 F^- (Clowes, unpublished)).

(i) *Transfer of colI from 58–161 to 58–161* is shown in Fig. 4, where transfer was

followed from a *colI*$^+$ strain to a *col*$^-$ strain, using four possible permutations of F$^+$ and *colI*$^+$ in the mating strains, mixed in equal concentrations. The control F$^-$ × F$^-$ curve (curve A), has the same shape as that shown for LFC in Figs. 2 and 3. The presence of *F* in the *colI*$^+$ strain (curve B) initially decreases the rate of transfer. After about 2 hr the rate increased to the value of the F$^-$ × F$^-$ control. In contrast, when *F* was present in the *col*$^-$ strain (curve C), there was an initial enhancement of transfer which progressively increased. Finally, when *F* was present in both strains (curve D), the initial rate paralleled that of the F$^-$ × F$^-$ control, but increased more rapidly, and after 2 or 3 hr was equivalent to the enhanced rate of transfer in the F$^-$ × F$^+$ mixture.

Table 3. *Duration of enhanced infectivity in HFC mixtures*

An HFC donor mixture was prepared by inoculation of 5×10^4 cells of a *colI*$^+$ *str-s* (519) with 10^6 cells of a *colI-r str-s* strain (547) into 5 ml. nutrient broth, incubating overnight, then diluting 1/20 in fresh broth and re-incubating for a further 90 min. (time 0). Incubation of this mixture was continued with a 1:1 dilution into fresh warm broth every 30 min. to maintain the population at about 10^8/ml. A recipient strain *colI-r str-r* (550) was grown overnight in broth, diluted 1 in 5 in fresh nutrient broth (10^8 cells/ml.) and held at 4°. At given times 1 ml. of HFC donor mixture was added to 1 ml. pre-warmed recipient culture and incubated for 10 min. at 37°. The mixture was then gently diluted 10^{-5} into buffer, incubated at 37° for 1 hr, and then recipient cells assayed for acquisition of *colI*$^+$.

Time of pre-incubation of HFC donor mixture (hr)	0	½	1	1½	2
Fraction of recipient colonies becoming *colI*$^+$	161/604	295/1475	194/1514	131/1306	78/1238
Degree of *colI*$^+$ transfer (%)	26·5	20·0	12·8	10·0	6·3
Time of pre-incubation of HFC donor mixture (hr)	3	4	5	6	
Fraction of recipient colonies becoming *colI*$^+$	20/934	61/1179	57/1476	42/1374	
Degree of *colI*$^+$ transfer (%)	2·1	5·2	3·9	3·0	

(ii) *Transfer of colI in 58–161 × w677 mixtures* occurred at a much slower rate in the absence of *F* than in the previous strain system. When *colI* was present in 58–161 F$^-$, transfer to w677 F$^-$ was only 1–3 %, even after 8 hr of mixed incubation, and did not exceed 20 % after overnight incubation. When *F* was present in either or both strains, *colI* transfer remained low and no significant differences were noted in either the rate of transfer or the final overnight value achieved. Nevertheless, in the 58–161 F$^+$ *colI*$^+$ × w677 F$^-$ *colI*$^-$ mixture, *F* transfer was normal, reaching 80–100 % after contact for 1 hr, by which time *colI* transfer was less than 0·1 %.

(iii) *Transfer of colI in Hfr × F*$^-$ *mixtures* was investigated by using Hfr Hayes (and A'Hfr) × 58–161 F$^-$. The effects of integrated *F* in either strain on *colI* transfer observed in this system (Fig. 5) were similar to those found for autonomous *F* shown in Fig. 4. When an Hfr strain was used as a *colI* donor, *colI* transfer was initially repressed (curve B), but after 4–8 hr increased to the value shown by the F$^-$ × F$^-$ mixture in curve A. The opposite effect was again observed in transfer of *colI* from F$^-$*colI*$^+$ to Hfr *col*$^-$ (curve C), when enhanced transfer ensued.

The effect of the F sex factor on colI transfer under HFC conditions

Under LFC conditions, *colI* transfer from either 58–161 F$^-$*colI*$^+$ or w677 F$^-$*colI*$^+$ to w677 F$^-$*col*$^-$ was less than 1 % even after contact for 5 hr, making observation of the effect of *F* on this transfer tedious and inaccurate in these systems. However

with the enhanced efficiency of HFC, the effect of *F* on *colI* transfer can be investigated in these mixtures. The kinetics of such transfer in a system using 58–161 newly infected with *colI* is shown in Fig. 6. In all mixtures, HFC conditions prevailed, and by 30 min. at least 60 % of the recipient w677 population had acquired *colI*. However the presence of *F* in the newly infected *colI*$^+$ strain diminished transfer within this period from 80 to 60 %, whereas when *F* was present in the recipient, transfer was increased to 85 % during this period. Very similar results were found with parallel mixtures of 58–161 strains used as both donor and recipient under HFC conditions, and in this system the overall transfer in all mixtures was higher, reaching 50–80 % after 15 min. and 75–90 % after 30 min.

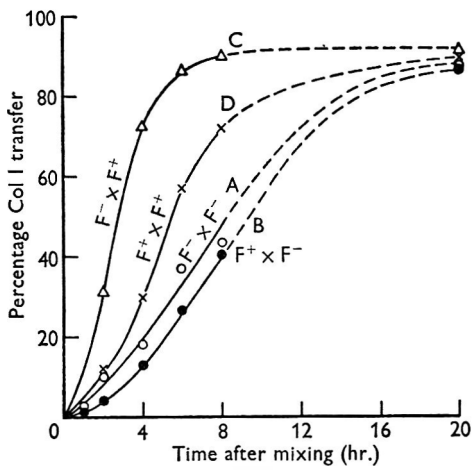

Fig. 4

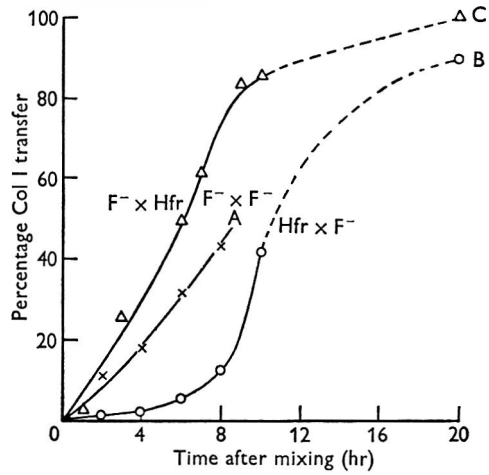

Fig. 5

Fig. 4. The effect of autonomous *F* on LFC transfer of *colI*. The curves show the transfer of *colI* in the following strains:

Curve A	F$^-$ × F$^-$; F$^-$ *colI*$^+$ *str-s* (519) × F$^-$ *colI-r str-r* (550)	○——○
Curve B	F$^+$ × F$^-$; F$^+$ *colI*$^+$ *str-s* (536) × F$^-$ *colI-r str-r* (550)	●——●
Curve C	F$^-$ × F$^+$; F$^-$ *colI*$^+$ *str-s* (519) × F$^+$ *colI-r str-r* (544)	△——△
Curve D	F$^+$ × F$^+$; F$^+$ *colI*$^+$ *str-s* (536) × F$^+$ *colI-r str-r* (544)	×——×

For details, see legend to LFC, Fig. 3. The transfer at each time is based on scoring between 400 and 1000 recipient clones for colicinogeny.

Fig. 5. The effect of integrated *F* (Hfr) on LFC transfer of *colI*. The curves show the transfer of *colI* in the following strains:

Curve A	F$^-$ × F$^-$; F$^-$ *colI*$^+$ *str-s* (519) × F$^-$ *colI-r str-r* (450)	×——×
Curve B	Hfr × F$^-$; Hfr *colI*$^+$ *str-s* (733) × F$^-$ *colI-r str-r* (550)	○——○
Curve C	F$^-$ × Hfr; F$^-$ *colI*$^+$ *str-s* (519) × Hfr *colI-r str-r* (730)	△——△

For details see Fig. 3. Points on curve are based on 400 to 1000 recipient colonies scored for *colI*$^+$.

The effect of colI on F transfer

(i) *The effect of colI transfer under LFC conditions on F transfer* when *colI* was present in the cross, 58–161 *colI*$^+$F$^+$ × w677 F$^-$ was negligible. Similarly, when *colI* was present in either strain of the mixture 58–161 F$^+$ × 58–161 F$^-$, *F* transfer was little affected, and was in all cases about 15 % ($\pm$1·5 %) after incubation for 3 hr, reaching about 25 % ($\pm$2·5 %) after 18 hr. Moreover, there appeared to be little linked transfer of the two factors from a F$^+$ *colI*$^+$ strain, some 15 % of the recipients

becoming F+ after 3 hr and about 10 % *colI*+, whereas only 2 % had acquired both characters to become F+ *colI*+. To investigate whether this co-transfer involved associated structures carrying both the *F* and *colI* factors, a second round of transfer was studied from several of these newly infected 58–161 F+ *colI*+ strains to

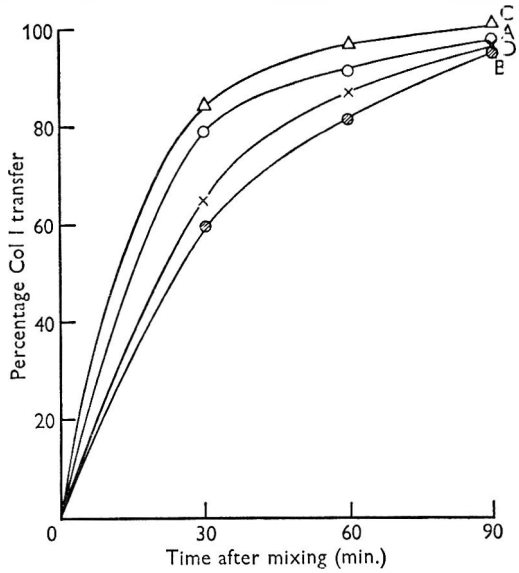

Fig. 6. The effect of autonomous *F* on HFC transfer of *colI*. The curves show the transfer of *colI* in the following strains:

		Donor	Intermediate	Recipient	
Curve A	F⁻ × F⁻;	F⁻ *colI*+ (519)	F⁻ (547)	F⁻ *colI-r str-r* (28)	○——○
Curve B	F+ × F⁻;	F+ *colI*+ (536)	F+ (535)	F⁻ *colI-r str-r* (28)	●——●
Curve C	F⁻ × F+;	F⁻ *colI*+ (519)	F⁻ (547)	F+ *colI-r str-r* (27)	△——△
Curve D	F+ × F+;	F+ *colI*+ (536)	F+ (535)	F+ *colI-r str-r* (27)	×——×

HFC cultures were prepared in the usual way and mixed at 1:1 ratio with 10^8 recipient cells/ml. Between 300 and 1000 recipient clones were tested for acquisition of colicinogeny at each time.

Table 4. *F transfer under HFC conditions*

In F+ donor or F⁻ recipient mixtures, usual HFC conditions were used. Log. phase cultures (10^8 cell/ml.) of F+ donor and F⁻ recipient were mixed 1:1 and incubated for 2 hr. Clones from the recipient cells were then isolated on streptomycin agar and tested for *F* and *colI*.

F+ donor (preparation)			% F transfer to recipient	% *colI* transfer to recipient
Donor	Intermediate	F⁻ recipient (preparation)		
F+ (535)	—	F⁻ *str-r* (550)	19·6	—
F+ *colI*+ (536)	F+ (535)	F⁻ *str-r* (550)	32·4	100
F+ *colI*+ (536)	F⁻ (547)	F⁻ *str-r* (550)	1	95
F+ (535)	—	HFC system:		
		F⁻ *colI*+ *str-s* (519) + F⁻ *str-r* (550)	40·5	100

w677 F⁻ *col*⁻ strains. In all crosses, *F* transfer achieved the expected value of 100 % in 3 hr, whereas *colI* transfer remained at 5–6 % and was similar to the transfer found with 58–161 F+ *colI*+ donors, in which the two factors had been introduced independently into the 58–161 strain.

(ii) *The effect of HFC on F transfer* was investigated by using F^+ and F^- strains newly infected with *colI*. Table 4 shows that *F* transfer from such a newly *colI* infected strain (line 2) was increased from about 20 to about 32 %, and a similar increase obtained when an F^- strain newly infected with *colI* was mixed with an F^+ *col*$^-$ strain (line 4).

The effect of colI on F$^+$ and Hfr fertility

The presence of *F*, either in the autonomous (F^+) or integrated (Hfr) state, in *Escherichia coli* K12 strains is shown by the ability of such strains to act as genetic donors, resulting in chromosomal transfer to a recipient strain. In addition, such genetic donors of either F^+ or Hfr type adsorb and plate a male-specific phage which is not adsorbed by F^- strains (Loeb, 1960; Dettori *et al.* 1961). F^+ *colI*$^+$ and Hfr *colI*$^+$ strains were found to plate the phage μ of Dettori *et al.* as efficiently as their non-colicinogenic counterparts. F^- strains did not plate this phage to any extent (<1 in 10^{11}), and F^- *colI*$^+$ strains were equally insensitive. Similar results were found whether the *colI* factor had been newly introduced into these strains, or when established colicinogenic strains were used as indicators.

The effect of *colI*, either as a newly introduced or as a stable factor, on the ability of F^+ and Hfr strains to act as chromosomal donors was studied in crosses with 58–161 F^+ and Hfr Hayes. In these crosses, transfer of the chromosomal markers *thr* and *leu* to the recipient w677 F^- was investigated (Table 5). In the F^+ cross, there was no apparent effect on *thr leu* transfer, and in the Hfr cross there was a threefold increase when *colI*$^+$ was present in the Hfr strain (this may, however, have been within the limits of experimental variation).

Table 5. *The effect of the presence, or new infection of colI on the transfer of the chromosomal markers thr and leu from Hfr and F$^+$ strains*

Five ml. of log.-phase cultures of F^+ (methionineless) or Hfr (prototrophic) strains, both *str-s*, were mixed with 5 ml. of log.-phase recipient cultures *thr leu str-r colI-r* (28) and incubated for 2 hr. Samples selected on streptomycin nutrient agar (recipient F^-) were then tested for colicinogeny. Prototrophic *str-r* recombinants were selected on streptomycin minimal medium plates: 100 recombinants from each cross being tested for *colI*$^+$.

Donor (preparation)		No. of *thr*$^+$ *leu*$^+$ recombinants ($\times 10^{-3}$)*	Fraction of *colI*$^+$ among recipient F^- (%)	Fraction of *colI*$^+$ among *thr*$^+$ *leu*$^+$ recombinants (%)
Donor	Intermediate			
F^+ (535)	—	3·12	—	—
F^+ *colI*$^+$ (536)	—	2·41	0·8	51·2
F^+ *colI*$^+$ (536),	F^+ (535)	3·52	44	100
Hfr (735)	—	1010	—	—
Hfr *colI*$^+$ (733)	—	3880	2·6	10
Hfr *colI*$^+$ (733)	Hfr (735)	3140	4	15

* Average of 3 plates.

Transfer of colI to recombinants

Table 5 shows that in the two F^+ mediated crosses, *colI* transfer to the recombinants was much increased as compared to the population as a whole. The possibility that *colI* was transferred to these recombinants as a chromosomal marker was therefore examined in a series of crosses with Hfr *colI*$^+$ strains which differed in

their 'origines' and directions of transfer, selecting in each instance for the same three classes of recombinants *pro*$^+$, *his*$^+$ and *try*$^+$. Table 6 suggests that irrespective of the marker selected, or the donor Hfr strain used, the transfer of *colI* was higher, the later the time of entry of the selected marker.

Table 6. *colI transfer in crosses of Hfr colI*$^+$ *strains with the same recipient strain* J 62 (*try, pro, his, str-r colI-r* F^-)

Log.-phase cultures of the Hfr strains (*colI*$^+$ *str-s*) and of the recipient 765 (*pro, his, try, T6-r, str-r, colI-r*), both at about 10^8 cells/ml., were mixed in equal volumes and incubated for 2 hr. At various times, samples were removed, infected with T6, 'blended', and recombinants selected, from which the time of entry of the marker was determined (Jacob & Wollman, 1961). After 2 hr, unblended samples were plated to select for recombinants which were tested for the acquisition of *colI*$^+$.

		Selected markers								
		histidine (*his*$^+$)			trytophan (*try*$^+$)			proline (*pro*$^+$)		
Hfr strain	Fraction of *colI*$^+$ among F^- recipients (*str-r*) (%)	Time of entry (*his*) (min.)	% rec	% *colI*$^+$	Time of entry (*try*) (min.)	% rec	% *colI*$^+$	Time of entry (*pro*) (min.)	% rec	% *colI*$^+$
AB 311 (763)	1·4	11	50	6	36	5	7	56	0·016	41
P 10 (764)	3·2	58	0·3	11	83	0·03	16	103	0·003	55
A'Hfr (733)	7·7	59	0·03	27	34	0·9	17	14	40	14

DISCUSSION

Readsorption of colicin in colicinogenic cultures

The indifference of a bacterial strain to the lethal action of a colicin can arise as a result of two distinct phenomena. The first, a mutation which involves the loss of a cell-wall receptor, is usually termed 'resistance', and is denoted genetically by the transition *col-s* to *col-r*, two allelic markers which have been mapped chromosomally with certain colicins (Frédéricq & Betz-Bareau, 1953; Jenkin & Rowley, 1955). The second is by acquisition of the colicin factor to produce a state of colicinogeny in the strain. Such colicinogenic (*col*$^+$) strains are assumed to adsorb colicin as readily as their non-colicinogenic (*col*$^-$) counterparts, but in some unknown way its lethal action is impaired, and the cell is said to be 'immune' (Frédéricq, 1956). The characteristics of resistance and immunity are independent, so that although a strain which is sensitive to colicin action must be *col*$^-$ *col-s*, an indifferent strain may be either resistant (*col*$^-$ *col-r*), immune (*col*$^+$ *col-s*), or both (*col*$^+$ *col-r*).

The amount of free colicin liberated by a culture of a colicinogenic strain appears, in the case of colicin I, to depend on whether or not this strain is genetically resistant to the colicin. In general, only a minority (10^{-3}–10^{-5}) of the cells of a colicinogenic culture release colicin (Ozeki *et al.* 1959). It is assumed that much of this colicin is adsorbed by the non-producing cells in a non-resistant *colI*$^+$ culture (*colI*$^+$ *col-s*), and is responsible for decreasing the amount of free colicin in culture filtrates as compared with that found in colicin-resistant (*colI*$^+$ *colI-r*) strains. Plate 1 shows this phenomenon, where a number of *colI*$^+$ cells of both *col-s* and *col-r* genotype were plated to form surface colonies, and after sterilization with chloroform vapour were overlayed with a sensitive (*col*$^-$ *col-s*) indicator. Small and large inhibition zones characteristic of the *colI*$^+$ *colI-s* and *colI*$^+$ *colI-r* strains respectively are visible.

However, where a *colI*$^+$ *colI-s* colony has grown within the normal inhibition zone of a *colI*$^+$ *colI-r* colony, the zone was less extensive, presumably due to adsorption of colicin by the *colI*$^+$ *colI-s* colony.

Sex factor activities of colI

Since the colicin I factor can effect its own transfer in the absence of the *F* factor, it may be regarded as a sex-factor, and this conclusion is implicit in its ability to mediate chromosomal transfer in Salmonella (Ozeki & Howarth, 1961) and in *Escherichia coli* (Clowes, 1961, 1963*b*). In F$^+$ and Hfr cells, the presence of the *F* factor results in the formation of a specific cell-surface component which has been revealed by its sensitivity to periodate treatment (Sneath & Lederberg, 1961), by its ability to adsorb and plate a specific phage (Loeb, 1960; Dettori *et al.* 1961) and by immunological methods (Ørskov & Ørskov, 1960). These activities may be due to the presence of a single surface component known either as 'mating substance' (Sneath & Lederberg), 'male-specific phage receptor' (Dettori *et al.*) or 'f' antigen (Ørskov & Ørskov), although a complete loss of one activity, such as phage adsorption, may not necessarily be accompanied by the loss of another, such as the ability to produce mating substance and to form recombinants (Clowes, 1963*b*). A similar mating substance may also be produced, in at least a small proportion of cells of a culture carrying the *colI* factor, permitting such cells to aggregate with cells of the complementary *colI*$^-$ surface. Such contacts may be seen as cell clumps when *colI*$^+$ and *colI*$^-$ cultures of *E. coli* are mixed, although no aggregation of cells is seen in pure cultures of either of the individual strains. However, this postulated *colI* mating substance does not appear to mask, and is distinct from, the *F* mating substance. This is shown by the ability of male-specific phage (μ) to plate on cells harbouring *F*, irrespective of whether these cells also carry *colI*, and by the inability of cells carrying *colI*, but not *F*, to plate this phage. These features are also seen in HFC cultures when most cells are able to conjugate and presumably to produce the specific *colI* surface. Furthermore, the surface change produced by *colI* differs from a similar change induced in *E. coli* cells by the presence of RTF (resistance transfer factor) which confers infectious transfer of multiple drug resistance (see Watanabe, 1963). The ability to plate male-specific phage is lost by both F$^+$ and Hfr strains when they also harbour RTF (Watanabe, Fukasawa & Takano, 1962).

The numbers of 'competent *colI* donors' of about 1 % found in *Escherichia coli* strains appears to be much greater than in Salmonella, where the corresponding value is about 1 in 6000 (Ozeki *et al.* 1962). This agrees with the finding of cell clumps in mixtures of *colI*$^+$ and *colI*$^-$ cultures of *E. coli*, whereas cell clumps are not observed in Salmonella except under HFC conditions (in *E. coli* also, a greatly enhanced aggregation of cells is seen in HFC cultures). Moreover, the rate of *colI* transfer in *E. coli* was higher, attaining between 7 and 10 % after contact for 2 hr, whereas in Salmonella at this time, less than 0·01 % transfer was observed (Ozeki *et al.* 1962). This difference probably accounts for the ability of stably *colI*$^+$ strains of *E. coli* to mediate chromosomal transfer (Clowes, 1961), whereas in Salmonella, chromosomal transfer is mediated only by cells newly infected with *colI* (Ozeki & Howarth, 1961).

Such newly infected cells become more efficient donors of *colI* (Stocker *et al.* 1963). The curves shown in Fig. 2 are best interpreted on the assumption that *Escherichia*

coli cells which have recently acquired colicinogeny are also enhanced as donors of *colI*. The transfer of *colI* may thus be regarded as a two part process: a first phase largely due to transfer from the original donor to the recipient, and a second phase predominating after this time, in which transfer occurs from newly infected recipients to non-infected recipients, leading to an exponential increase in the numbers of cells which acquire colicinogency and termed 'epidemic spread' (Ozeki *et al.* 1962). Under these circumstances optimal transfer obtains when the donor:recipient ratio is low, so that recipient to recipient contacts will be frequent and therefore favour the rapid initiation of this epidemic spread. The transfer of *colI* under high-frequency conditions (HFC) shown in Fig. 3, supports the conclusion that cells newly infected become highly efficient as donors of *colI*. The transfer appears more rapid in *E. coli* than in Salmonella, and transfer of *colI* to as many as 90 % of the recipients may occur within the first hour. The duration of this high infectivity appears to be about 4 to 6 generations, and is similar to Salmonella (Stocker *et al.* 1963).

The effect of F on colI transfer

The effect of *F* on *colI* transfer is readily interpretable when the kinetic curves shown in Fig. 4 are considered as a two phase process made up of an initial period during which donor:recipient transfer occurs, after which the recipient:recipient transfer predominates. The presence of *F* in the *colI*$^+$ donor decreases this early transfer, from which it is concluded that the *F* factor is epistatic to the *colI* factor and in some way suppresses its activity. This epistasis could be due to suppression or masking of the proposed *colI* specific mating surface by that of the *F* mating surface. Furthermore, *colI* transfer does not occur through *F*-mediated contacts, since *F* transfer itself may occur at a rapid rate in the absence of significant *colI* transfer. Transfer of *colI* at a decreased rate is suggested to occur only from those F$^+$ cells which do not produce the *F* mating surface, and which are known to appear as F$^-$ phenocopies (Lederberg, Cavalli & Lederberg, 1952) as cells pass from the logarithmic phase to the stationary phase of growth. After the first few hours, recipient:recipient, *F*$^-$*colI*$^+$ × *F*$^-$*colI*$^-$ transfer seems likely to account for the shape of the curve, which is similar to that of the control curve, the recipient *col*$^-$ strain being in both instances *F*$^-$.

In contrast, the presence of *F* in the recipient strain (*F*$^-$ × *F*$^+$) appears to enhance early transfer (Fig. 4, curve C). Here the *col*$^-$ strain produces the *F* mating substance, which may form *F* mediated contacts with the *F*$^-$*colI*$^+$ strain. Such contacts would be likely to hold cells of the two populations together, so as to either stabilize any specific *colI*-mediated contacts made by the F$^-$*colI*$^+$ cells or increase the probability that such contacts would be formed. The enhancement of transfer found in the early stages is preserved in the later recipient:recipient phase, from which it is inferred that the epistasis of *colI* by *F*, suggested to exist in established *colI*$^+$ strains, does not occur to the same extent in cells newly infected with *colI*. In F$^+$ × F$^+$ mixtures (Fig. 4, curve D), initial transfer appears to be enhanced, but the increase is not so marked as in curve C. Here the putative F$^-$ phenocopies in which *colI* is expressed have an increased probability of forming specific *F*-mediated contacts with the *col*$^-$ strain which is also F$^+$. The effect of this *F* directed mating is suggested to outweigh the reduction in the total numbers of *colI*$^+$ donors due to the presence of *F*, since this early transfer is generally greater than that in the F$^+$

col$^+$ × F$^-$ *col*$^-$ cross. The latter part of the transfer kinetics is likely to resemble that in the F$^-$ × F$^+$ mixture due to the emergence of a similar F$^+$ *col*$^+$ × F$^+$ *col*$^-$, recipient:recipient transfer process.

In Hfr strains (Fig. 5), similar interpretations hold, although there are indications that epistasis is more marked (Fig. 5, curve B). In curve C there is an enhanced early transfer of *colI* from an F$^-$ to an Hfr strain, which occurs under conditions in which a presumptive chromosomal transfer is proceeding in the majority of cells in the opposite direction. There is however no evidence to support the idea that the same pair of cells may at the same time be able to transfer chromosome in one direction through one contact, and colicin factor through another.

The results in HFC systems (Fig. 6) support the idea that the *F* epistasis of *colI* is largely overcome in newly-infected cells. The effect persists to a small extent in decreasing the efficiency of *colI* transfer from F$^+$ strains, although the majority of cells are still able to transfer *colI* very rapidly. Similarly, when *F* is present in the recipient, enhanced transfer obtains, so that *F* contacts appear to be able to supplement even this high degree of *colI*-mediated contacts.

The effects of colI on F-mediated activities

No effects of *colI* on F-mediated functions are found under LFC conditions; the expression of the *F* mating surface is little affected as judged either by the plating efficiency of male-specific phage, or the fertility of these strains. The apparent inhibition of *colI*-mediated conjugation by *F* thus appears to be true epistasis, since there is no suppression in the opposite sense. However, under HFC conditions *F* transfer may actually be *increased* after new infection with *colI* of either the F$^+$ or the F$^-$ cell. In the latter case a high degree of *colI*-mediated contacts made by *F*$^-$ *col*$^+$ cells is likely to assist and stabilize the formation of *F*-mediated contacts by the F$^+$ *col*$^-$ strain. For the enhancement of *F* transfer by F$^+$ cells newly infected with *colI*, the possibility exists that either the *F* factor can be transferred through a *colI*-mediated contact, or what seems more likely in view of the fact that judged by phage μ plating the *F* surface is unimpaired even in HFC mixtures, that the sites of both the *colI* and *F* specific mating surfaces are restricted to only part of the cell surface, and are topologically distinct. Similar conclusions have been drawn in previous publications (Jacob, Brenner & Cuzin, 1963; Clowes, 1963*b*).

Genetic classification of colicin factors

The transfer of *colI* to recombinants arising from both F$^+$ and Hfr crosses is greater than to the recipient population at large (Tables 5, 6). Selection for recombinants selects those cells which have been in contact for longer than average periods to allow time for chromosomal transfer; this is slow, the complete transfer taking 110–120 min. (Jacob & Wollman, 1961). It is suggested that there is a greater probability of *colI*-mediated contact and transfer in cells united over these long periods.

This idea is supported by the results of the three Hfr crosses shown in Table 6. After 2 hr, transfer of *colI* to the F$^-$ recipients varied from 1·4 to 7·7 %, depending on the Hfr strain used. At this time, transfer to all recombinant classes was higher, even though some markers were proximal and entered early. Moreover, in each

cross, irrespective of the marker in question, or the Hfr donor strain, the transfer to recombinants was greater as the time of entry of the selected marker was later. Thus, in selecting for *his*$^+$, *try*$^+$ and *pro*$^+$ recombinants from HfrAB311, where the respective times of marker entry were around 11, 36 and 56 min. (such times representing the minimum time that the donor and recipient cells have been in contact) transfer of *colI* was 6, 7 and 41 %, respectively. With HfrP10, where this particular order of entry was preserved, but where the entry of each marker occurred about 50 min. later, these frequencies were, respectively, 11, 16, and 55 %. In contrast, with Hfr Hayes, where the order of entry is reversed, *pro*$^+$ entering at 14, *try*$^+$ at 34, and *his* at 59 min., the proportion of each of these recombinant classes acquiring *colI* were 15, 17 and 27%. These results show no correlation of *colI* transfer with any particular chromosomal segment, but support the idea that selection for distal (late) markers selects for cell pairs held together by Hfr to F$^-$ surface interactions for periods longer than the time of entry of the marker in question, so increasing the period over which extra-chromosomal *colI* transfer may take place, presumably through topologically distinct *colI*-mediated contacts. Alternatively, it is possible that *colI* is attached together with *F* to the terminal chromosomal segment, in which case selection for later markers would increase the probability that the distal end of the chromosome will enter. However 'interrupted' matings, when donor and recipient are separated after periods of contact of 30 min. or less, invariably give rise to some *col*$^+$ recombinants among the early markers selected. This alternative would therefore require that, in addition to the transfer of *colI* on the tail of the chromosome, it is also transferred extra-chromosomally, and for this reason seems unlikely. Moreover, there is no indication of *F* and *colI* linkage in transfer experiments from F$^+$ *colI*$^+$ cells. Both factors are therefore suggested to exist as independent extra-chromosomal elements in F$^+$ cells, the *colI* factor maintaining this situation when the *F* factor is chromosomally integrated in Hfr strains.

Since these experiments were initiated, experiments reported by Nagel de Zwaig, Anton & Puig (1962), suggest that *colI*, *colV* and *colE2* may be transferred as extra-chromosomal elements. *ColE1* is the only colicin factor for which a chromosomal site has been claimed (Alfoldi, Jacob, Wollman & Mazé, 1958) from which it was suggested that all colicin factors were episomes (Jacob, Schaeffer & Wollman, 1960). This was re-investigated by Clowes (1963*a*), and from more extensive experimental evidence it was concluded that *colE1* was in fact transferred extra-chromosomally, with the suggestion that all colicin factors are best considered as plasmids (Lederberg, 1952) having only a cytoplasmic existence, until a direct demonstration of chromosomal location permits their recognition as episomes.

The assistance of Mr E. E. M. Moody in the preparation of many strains and with some experiments is gratefully acknowledged. Some of this work has been submitted by one of us (M.M.) in partial fulfilment for the Degree of Doctor of Philosophy in the University of London, who wishes to acknowledge the receipt of a Canteens Services Trust Fund (Australia) Scholarship, and the generous use of facilities extended by Dr W. Hayes, F.R.S., and the Medical Research Council.

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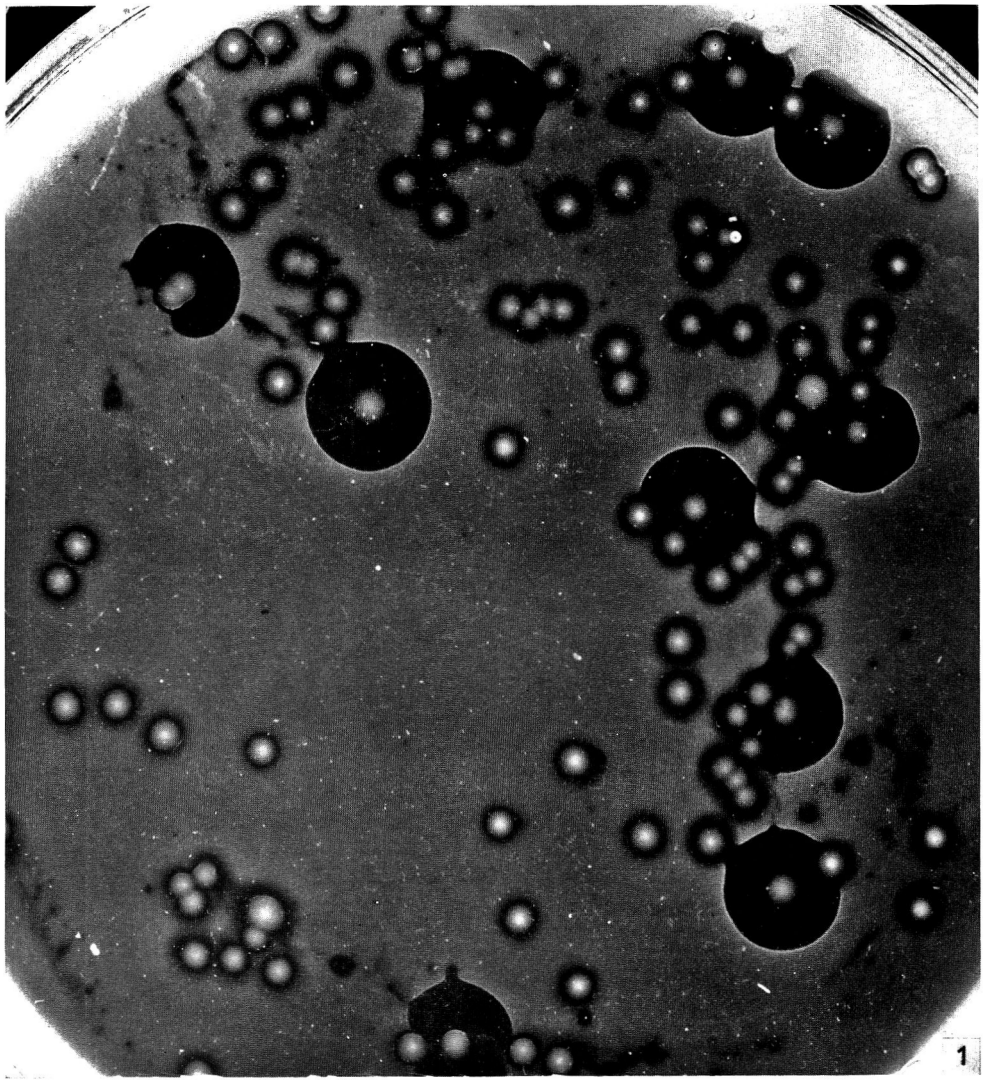
EXPLANATION OF PLATE

Fig. 1. Relative inhibition zone sizes of *colI*$^+$.*colI-s* and *colI*$^+$.*colI-r* strains. A mixture of *colI*$^+$.*colI-s* (519) and *col*$^+$ *colI-r* (554) was diluted and plated on nutrient agar to produce isolated surface colonies. After overnight incubation, the plate was treated with chloroform vapour, then over-layered with about 10^8 cells of a *colI-s* indicator (511) in 2·5 ml. soft agar and re-incubated over-night.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J. gen. Microbiol. (1964), **36**, 385–392
Printed in Great Britain

The Regulation of Colicin Synthesis and Colicin Factor Transfer in *Escherichia coli* K12

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(*Received* 20 *March* 1964)

SUMMARY

Cells of *Escherichia coli*, newly infected with the colicin I factor (*colI*), showed an enhanced efficiency of transfer of this factor (HFC), and were also more likely to undergo lethal colicin synthesis, than were stably colicinogenic cells. Up to 20% of the cells of stably *colI*$^+$ strains were induced to produce colicin by ultraviolet irradiation, and from such irradiated cultures transfer of the *colI* factor occurred more efficiently. To account for these results, it is proposed that the *colI* factor exists as an autonomous non-chromosomal genetic element which sets up its own system of self-regulation within cells of stably colicinogenic strains.

INTRODUCTION

Colicin production in stably *colI*$^+$ strains of *Salmonella typhimurium* was demonstrated by Ozeki, Stocker & de Margerie (1959) who observed the release of colicin from single bacteria as minute clearings termed 'lacunae' in a lawn of colicin-sensitive cells. An enhancement in the number of these 'lacunae' following ultraviolet (u.v.) irradiation of colicinogenic strains was shown by Ozeki (1960) and ascribed to the induction of lethal colicin synthesis in an increased number of cells of a *col*$^+$ population. In *Salmonella typhimurium*, cells newly infected with the *colI* factor are highly efficient in the further transfer of this factor (Stocker, Smith & Ozeki, 1963). It was suggested (Smith & Stocker, 1962; Stocker *et al.* 1963) that in stably colicinogenic cells, the *colI* factor exists chromosomally, but in a small minority of cells it may take up a cytoplasmic state, in which event it becomes capable of infectious transfer at low frequency (low-frequency colicinogeny transfer, LFC). Newly transferred *col* factors are suggested to persist for some time in this cytoplasmic state, during which time the efficiency of further *colI* transfer remains high, giving rise to a system of high-frequency colicinogeny transfer (HFC) before the proposed stable chromosomal condition is re-established. We have previously shown the existence of high-frequency colicinogeny transfer (HFC) in *Escherichia coli* K12 strains newly infected with *colI* (Monk & Clowes, 1964). In the present paper we report the incidence of colicin-producing cells ('lacunae') in both LFC and HFC cultures of *E. coli* K12, together with related studies on the induction of lacunae and *colI* transfer following u.v.-irradiation of stably *colI*$^+$ *E. coli* K12 cultures. An alternative hypothesis to account for HFC and colicin induction based on a system of self-regulation of non-chromosomal *colI* factors is proposed.

METHODS

Media and bacterial strains. Those used in this study are described in Monk & Clowes (1964). To avoid re-adsorption of colicin, all the *colI*$^+$ strains used were also *colI-r*. In addition, derivatives of a non-lysogenic (λ^-) strain, W1655 (Lederberg & Lederberg, 1953) were used in u.v.-irradiation experiments to prevent lysis and death arising from induction of the λ prophage. Some W1655 derivatives were isolated from a strain cured of *F* by the method of Hirota (1960) by using acridine orange. One of these strains (59), an F$^-$ derivative of W1655, made resistant to colicin I (*colI-r*) and colicinogenic (*colI*$^+$), was used in these studies.

Assay of colicin production by single cells (lacunae). To 2·5 ml. soft agar, seeded with 0·1 ml. of an overnight *col-s str-r* indicator strain, was added an inoculum of 10^7 cells of a *colI*$^+$ *str-s* strain, and the agar layered over a streptomycin nutrient agar plate. After incubation for 5–6 hr, small clearings (lacunae) in the confluent indicator lawn were visible. Alternatively using a *colI*$^+$ strain which was *str-r*, several drops of chloroform were added to the broth culture, which was then incubated for 10 min. before adding diluted samples to the soft agar indicator mixture.

Ultraviolet (u.v.)-irradiation was achieved using a Hanovia 15 W. 'germicidal' lamp emitting 95 % of its radiation at 2537 Å. Cells were irradiated at 50 cm. from the lamp, the intensity at this distance being 5 ergs/mm.^{2}/sec. Logarithmic-phase *colI*$^+$ bacteria, grown in nutrient broth, were centrifuged at low speed (3000 rev./min.), and the supernatant fluid replaced with phosphate buffer (0·5 M; pH 7·5) to produce a cell concentration of 1 to 2×10^8/ml. Five ml. samples were u.v. irradiated in 9 cm. Petri dishes for the desired time with gentle agitation. The irradiated cell suspension was then rapidly transferred to an equal volume of pre-warmed, double-strength nutrient broth and incubated with aeration in the dark.

Other colicin techniques were as described previously.

RESULTS

Lacunae production in K 12 colI$^+$ *strains*

The small clearings (lacunae) produced by *colI*$^+$*str-s* Salmonella cells in a confluent lawn of *colI*$^-$*str-r* indicator bacteria on a streptomycin overlay plate were ascribed by Ozeki *et al.* (1959) to the release of colicin by single bacteria. Lacunae produced by *Escherichia coli* K12 *colI*$^+$ strains form just visible clearings in the indicator lawn and are difficult to score with precision, there being considerable variation in their size and clarity. Triplicate or quadruplicate platings were independently scored on at least two occasions, and differences were only regarded as significant when they exceeded tenfold. The frequency of lacunae observed in stably *colI*$^+$ cultures is generally about $1/10^5$ cells (a variation from about 3 in 10^5 to 3 in 10^6 cells). These clearings were confirmed as lacunae since they were not produced with cell-free filtrates, and they occurred with a frequency directly proportional to the numbers of cells plated. Moreover, they were not transferred by subculture, and were thus non-infective, and they were not detected when a *colI-r* strain was used as an indicator, nor when the *col-s* indicator was plated in the absence of a *colI*$^+$ inoculum.

Induction of colicin I synthesis by ultraviolet-irradiation

Although induction of colicin E1, E2 and B by u.v.-irradiation has been reported in *Salmonella typhimurium* by Ozeki (1960), induction of colicin I was not achieved. A *colI*$^+$ *colI-r str-s* strain of *Escherichia coli* K12, non-lysogenic for phage λ, was irradiated for various times and assayed for lacunae production after 100 min. of post-irradiation incubation. As the dose of u.v.-radiation was increased, the numbers of lacunae were increased, reaching a plateau after irradiation for 60 sec. when about 20 % of the cells formed lacunae. The time of 60 sec. was used in further experiments in which the number of lacunae arising after various times of post-irradiation incubation were assayed; the results are shown in Fig. 1. At this dose,

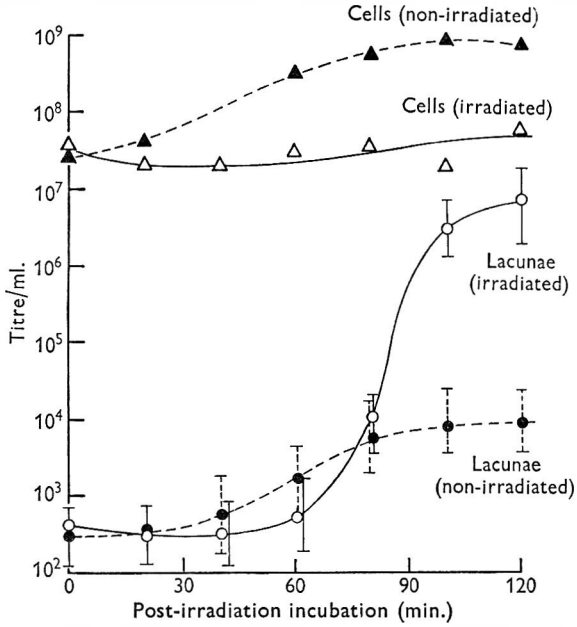

Fig. 1. Viable count and lacunae of a *colI*$^+$ culture after u.v.-irradiation. A log.-phase culture of *colI*$^+$ *colI-r str-s* λ^- bacteria (strain 59) was centrifuged and resuspended at 10^8 cells/ml. in buffer. A 5 ml. sample was removed to 5 ml. double-strength nutrient broth to serve as a control unirradiated culture. A further 5 ml. was then u.v.-irradiated for 60 sec. at 50 cm. from the u.v. source, and then similarly diluted into 5 ml. double-strength nutrient broth. Both samples were then incubated at 37° with aeration and at various times further samples were removed and assayed on nutrient agar for viable count, and streptomycin nutrient agar with a *col-s str-r* indicator for the numbers of lacunae. ▲ - - - ▲, Viable count of non-irradiated culture: △——△, viable count of u.v.-irradiated culture: ● - - - ●, lacunae in non-irradiated culture: ○——○, lacunae in u.v.-irradiated culture.

the irradiated cells did not show any marked decrease in viable count, although cell-division was considerably delayed. The number of lacunae remained constant at the uninduced value until about 60 min. after irradiation, when a sharp increase occurred, reaching a maximum at 120 min. of about 10^7 lacunae/ml., and corresponding to an induction of about 20 % of the total number of irradiated cells. At this time the amount of free colicin in the cell-free filtrate showed an increase of about 30-fold.

Enhanced transfer of colI from irradiated colI⁺ cultures

A culture of a stably *colI*⁺ strain was u.v.-irradiated for various periods under the conditions optimal for induction, and samples of the irradiated culture mixed with equal numbers of log.-phase *colI-r str-r* recipient cells in double-strength broth, and re-incubated for 2 hr. The mixed culture was then diluted and the recipient cells assayed for acquisition of *colI*; the results are shown in Table 1. After irradiation for 10, 20 or 30 sec., a small but reproducible increase in *colI* transfer resulted.

Table 1. *Relative increase in colI transfer after u.v.-irradiation*

Log.-phase cultures of strain 59 were centrifuged and resuspended in buffer at 10^8 cells/ml. Samples (5 ml.) were u.v.-irradiated for the times shown, then 1 ml. samples mixed with 1 ml. log.-phase cells of a culture of 58–161 λ^+ F^- *colI-r str-r* (550) in double-strength nutrient broth at 10^8 cells/ml. Mixtures were incubated for 2 hr and *str-r* cells selected and tested for *colI*⁺. Three hundred to 1000 clones were scored at each time and expressed relative to the numbers of *colI*⁺ found after contact with unirradiated colonies as 100. The absolute value ranged from 5 to 20 % transfer.

	Time of u.v.-irradiation (sec.)					
Exp.	0	10	20	30	40	60
	Relative no. of *colI* transfer					
1	100	180	230	220	200	170
2	100	—	180	—	160	110
3	100	310	360	—	110	50
4	100	180	220	210	150	110
5	100	140	150	130	100	80
6	100	200	200	170	120	100
7	100	150	150	180	150	100

This increase was from 1·3- to 3·6-fold, calculated on the numbers of *colI*⁺ input cells. However, if the increase in infectivity is assumed to occur at the same time as the onset of induction, namely about 90 min., the non-irradiated control cells have multiplied by a factor of about 20. The relative transfer per viable cell might therefore be enhanced by as much as 30- to 70-fold as a result of u.v.-irradiation.

Enhanced lethal colicin synthesis in HFC cultures

When incubated overnight preparations of HFC were diluted 1/20 in broth to prepare standard HFC 'donor mixtures', it was found 15 min. later that the numbers of 'intermediate' cells which were recovered as viable colonies had decreased to about 50 %. Similarly, when a *colI-r* recipient strain was mixed with such a HFC donor mixture, only 50–70 % of the recipient cells survived as colony-formers when plated 15 min. after mixing. Since both populations which showed this decrease were non-adsorbing for colicin I, the observed lethal effects appear to be due to a transfer of the *colI* factor, rather than to any direct colicin action. The number of lacunae-forming cells and free colicin were therefore determined in a HFC donor mixture and compared with similar assays in a LFC donor culture; the results are shown in Table 2. It can be seen that under HFC conditions the numbers of lacunae increased more than 100-fold, with an accompanying 16-fold increase in the cell-free colicin.

Table 2. *Lethal colicin synthesis in HFC and LFC cultures*

HFC preparation: 5×10^4 cells of donor *colI*$^-$ *str-s* (519), and 10^6 cells of intermediate *colI-r str-s* (547) were mixedly inoculated into 5 ml. nutrient broth, incubated overnight, diluted 1 in 20 and then incubated for 90 min. with aeration. For the control, the donor strain was mixed with the strain *colI*$^+$ *colI-r str-s* (559) and cultured in the same way. Samples were removed and (i) diluted and plated with *colI-s str-r* indicator in 2·5 ml. soft agar on streptomycin nutrient agar for numbers of lacunae: (ii) centrifuged, and doubling dilutions of supernatant fluid spotted on a streptomycin nutrient agar indicator plate for colicin titre: (iii) mixed with log.-phase recipient *colI-r str-r* cells (550) at a ratio of 1:1 and incubated for 1 hr. The proportion of *colI*$^+$ recipient cells was determined by triple overlay on streptomycin nutrient agar.

System	Viable cells/ml.	Lacunae/ml.	Limiting colicin titre	Transfer *colI* to recipient (%)
Newly infected *colI*$^+$ (HFC)	10^8	5×10^5	1/16	98
Stably *colI*$^+$ (LFC)	2×10^8	3×10^3	1	4·2

DISCUSSION

In *Escherichia coli*, cells newly infected with *colI* become highly efficient donors of this factor and transmission to a non-colicinogenic recipient is increased from about 1 % (LFC) to about 90 % (HFC) within the first hour (Monk & Clowes, 1964). The duration of this enhanced infectivity is about four to six generations, similar to the HFC condition first described in *Salmonella typhimurium* by Stocker *et al.* (1963). It has also been shown that an increase in the infectivity of a *colI*$^+$ culture is accompanied by an increase in the numbers of *colI*$^+$ cells which are able to form unions when mixed with non-colicinogenic recipient organisms. These unions may be observed by mixing *colI*$^+$ cells, which have been stained by the uptake of a tetrazolium dye, with unstained *col*$^-$ cells, whereupon pairing of *col*$^+$ and *col*$^-$ cells, or more frequently clumps including a number of both types of cells, may be seen. Such pairing or clumping is not seen in cultures of either strain independently. Stably *colI*$^+$ cultures of *E. coli*, which can transfer *colI* to about 7–10 % of recipient cells in 2 hr, are able to form a small proportion of cell clumps (about 5 %), whereas in HFC *E. coli* mixtures, a much higher proportion of the cells (50–100 %) are seen in clumps (Monk & Clowes, 1964). *S. typhimurium* HFC cultures (transmitting 50 % *colI* in 1 hr) also form clumps, but no pairing or association of cells is seen under LFC conditions, when the transfer in the first 2 hr is less than 0·01 % (Ozeki, Stocker & Smith, 1962). Increased infectivity would thus seem to be due, in part at least, to an increase in the numbers of *colI*$^+$ cells which can form stable contacts with *colI*$^-$ cells. These contacts are likely to result from an increase in the numbers of cells which form a specific *col*-directed conjugating cell surface (possibly a *col*-conjugating antigen similar to that produced by the presence of the fertility factor *F*) or an increase in the amounts of such conjugating antigen produced by most cells. Increased *colI* infectivity might also be due to an increase in the numbers of *colI* factors per cell, increasing the probability of *colI* transfer per contact. Recent experiments by Dr H. Ozeki (personal communication) on the 'suicide' of ^{32}P-labelled *col* factors after transfer indicate that there is a rapid multiplication of these factors within the newly infected cells.

In a previous publication (Monk & Clowes, 1964) we concluded that *colI*, like other colicin factors such as *colE1* (see Clowes, 1964), appears to be transferred

extra-chromosomally, and no evidence was found to suggest a chromosomal location. The multiplication and activity of a stable cytoplasmic element or plasmid (Lederberg, 1952), which is also a potentially lethal agent, yet whose lethality is seldom expressed, must be regulated in some way. Such regulation in the case of non-chromosomal elements is likely to be self-imposed and may, by analogy with other regulated and potentially lethal systems such as temperate phage, be due to the presence of a cytoplasmic repressor. This picture of the *colI* factor as a self-regulating plasmid, also independently suggested by Clark & Adelberg (1962), appears to be more compatible with the data shown in the present paper than the alternative model proposed by Smith & Stocker (1962).

In stable *col*$^+$ strains, the activities of the *colI* factor (which includes lethal synthesis of colicin, and the ability to form a conjugating cell surface) would be repressed in most cells. In a small proportion of *colI*$^+$ cells, this repression breaks down and lethal synthesis results. In other cells, de-repression might lead to an enhancement of non-lethal activities, such as the synthesis of the *col*-directed surface 'antigen' (and possibly of the numbers of *col* factors existing in the cell), which would allow these cells to conjugate and transfer *col* factors, or in other words to become 'competent donors' of *colI*, either without lethal colicin synthesis, or before lethality prevented transfer. This repression would appear to be less stringent in *Escherichia coli* than in *Salmonella typhimurium*, resulting in about a hundred fold greater number both of competent donors and of cells which proceed to lethal synthesis. (In *E. coli*, 'competent donors' are about 1 % and lethal synthesis is observed in about 1 in 10^5 cells, whereas in *S. typhimurium*, competent donors are about 0·02 % and lethal synthesis is found in less than 1 in 10^7 cells; Ozeki *et al.* 1962).

Transfer of the *colI* factor in the absence of its repressor would lead to a period of de-repression in newly infected cells. Such cells would themselves then have an increased probability of *colI* transfer, which could account for the epidemic spread of *colI*, giving rise to HFC donor mixtures under the appropriate conditions. De-repression in such newly infected cells would also be likely to have lethal consequences. This lethality is demonstrated when overnight HFC mixtures are subcultured, when extensive *colI* transfer to previously non-colicinogenic (intermediate) cells is occurring. This loss of viable cells is paralleled by the high incidence of lacunae-producing cells in such cultures (Table 2). When HFC donor mixtures are mixed with recipients to which *colI* is transferred to 80 % of the survivors after 30 min., this is also accompanied by lethal effects in the recipients, and 50–70 % do not survive 15 min. after mixing.

Such lethal effects following infection with *colI* are parallel to those subsequent to infection with temperate phage. Here infection may result in either lethal synthesis of phage, with consequent death of the cell (productive infection) or the establishment of a stably-lysogenic cell (reductive infection, Lwoff, 1953). Although in some instances, particularly the well documented phage λ system (Jacob, 1960), such lysogeny can be shown to be due to the integration of the phage genome as a prophage at a particular site on the bacterial genome, in other systems the phage genome may persist for some time in the cytoplasm without lethal effects (Luria, Fraser, Adams & Burrous, 1958). Moreover, even in the case of those phages in which prophage integration occurs, mutants may be isolated, such as phage λb2,

which can no longer integrate, but retain the ability to set up an immune 'abortive' lysogenic state (Zichichi & Kellenberger, 1963). With other phages (particularly those capable of 'generalized' transduction, such as P1 or 363), it has not so far been possible to establish a chromosomal location (Jacob & Wollman, 1958), and they too may exist as plasmids, capable of establishing an immune, stably-lysogenic state without integration.

The destruction of a cytoplasmic repressor has been suggested to account for induction of prophage to lethal synthesis following u.v.-irradiation of a lysogenic strain (Jacob & Monod, 1961). In *colI*$^+$ cultures of *Escherichia coli*, u.v.-irradiation also results in greater than a thousand-fold increase in the numbers of cells which proceed to lethal synthesis (Fig. 1). Such induction is accompanied by a small but reproducible increase in the infectivity of *colI*, both effects being ascribed to the destruction of a regulation system. Similar effects on enhanced transfer after u.v.-irradiation of strains which harbour RTF have been suggested (Watanabe & Fukasawa, 1961) to be due to a displacement of the RTF element from the chromosomal to the cytoplasmic state. De-repression of an autonomous plasmid is an alternative hypothesis that may be equally valid in this system.

Some of this work has been submitted by one of us (M.M.) in partial fulfilment of the requirements for the Degree of Doctor of Philosophy in the University of London, who wishes to acknowledge the receipt of a Canteens Services Trust Fund (Australia) Scholarship and the generous use of facilities extended by Dr W. Hayes, F.R.S., and the Medical Research Council.

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J. gen. Microbiol. (1964), 36, 393–403
Printed in Great Britain

Penetration of Substances into Cold-Shocked Bacteria

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(*Received* 31 *March* 1964)

SUMMARY

The death-rate of washed exponential phase *Aerobacter aerogenes* chilled in saline phosphate buffer (pH 6·5) at 0° was increased by ribonuclease (RNase) but not by deoxyribonuclease, trypsin, pepsin or lysozyme; none of these enzymes had any immediate effect on the viability of similar bacterial suspensions at 20°. Leakage products from chilled *A. aerogenes*, Mg^{2+} and, to a smaller extent, 0·3 M-sucrose, antagonized the lethal effect of RNase on chilled organisms. RNA degradation occurred when bacteria were chilled and then incubated in fresh diluent at 37°; organisms exposed to RNase during chilling degraded RNA at 20–25° when the rate of auto-degradation of RNA was low. As the salt content of the environment was decreased, the amount of RNase adsorbed by the bacteria and its lethal effect increased at both 0° and 20°; in distilled water RNase was more lethal at 20° than at 0°. Anilino-naphthalene-8-sulphonate penetrated into bacteria chilled in buffer containing this dye. Acid or alkali accelerated the death rate of bacteria to greater extents at 0° than at 20°. RNase increased the lethal effect of freezing and thawing on a population from a continuous culture and augmented subsequent degradation of RNA.

INTRODUCTION

The lethal effect of chilling on certain bacteria, which is called 'cold shock' (Sherman & Albus, 1923; Hegarty & Weeks, 1940; Gorrill & McNeil, 1960), may be due to interference with bacterial permeability control mechanisms (Meynell, 1958). Evidence for this view is that leakage of normal endocellular constituents occurs when susceptible bacteria are chilled (Strange & Dark, 1962; Strange & Ness, 1963). Evidence that substances penetrate from the environment into chilled bacteria to a greater extent than into unchilled bacteria would provide further support for this hypothesis. The present paper presents evidence that RNase, anilino-napthalene-8-sulphonate, hydrogen ions and hydroxyl ions more readily enter bacteria subjected to cold shock or freezing and indicates that the permeability damage is, in the absence of RNase, sometimes reversible.

METHODS

Organism and cultural conditions. Strain NCTC 418 of *Aerobacter aerogenes* was grown at 37° in shaken flasks (2 l.) containing the mannitol-limiting defined medium (100 ml.) previously described (Strange, Dark & Ness, 1961) seeded with 1–4 ml. of a fully grown (18–20 hr) culture (about 10^{10} viable bacteria/ml.) in the same medium. Bacteria growing exponentially were harvested after growth for 2–4 hr.

Viability determinations. The % viable bacteria in a suspension was determined

microscopically with dark-ground illumination after slide culture on the rich medium described by Postgate, Crumpton & Hunter (1961) except that glucose (0·5 %, w/v) replaced glycerol (0·2 %, w/v). In a few experiments the results were checked by plate counts; samples (0·2 ml.) of a suspension suitably diluted in saline phosphate buffer (pH 6·5) were spread on each of 3 or 5 plates of Oxoid Plate Count Agar (from Oxo Ltd.) and the colonies counted after incubation for 24 hr at 37°.

Chilling. Bacteria separated from a culture by centrifugation were washed once or twice with diluent and resuspended at a suitable concentration in the same diluent. Washed suspensions were chilled by dilution (1/10–1/50) into diluent held in a temperature controlled bath at 0°. Saline phosphate buffer diluent was prepared from a stock solution containing 2·2 M-NaCl and appropriate concentrations of $K_2HPO_4 + KH_2PO_4$ (0·4 M $-PO_4$; pH 6·5); just before use the stock solution was diluted with fresh glass-distilled water either 1/20 or more usually 1/40 ('dilute saline phosphate buffer' contained 0·055 M-NaCl and 0·01 M-PO_4) and filtered through well-washed Oxoid membrane filters (grade A.P.; Oxo Ltd.).

Incubation of chilled bacteria. Usually bacteria in a sample of chilled suspension (10–20 ml.; equiv. 0·4–0·5 mg. bacterial dry wt./ml.) were quickly sedimented in the chilled head of an M.S.E. Angle-13 high-speed centrifuge, rinsed twice with cold diluent (gently poured on and off the pellet) and resuspended in aerated diluent at 25° or 37°.

Freezing. Bacteria were frozen in liquid nitrogen and thawed, with the techniques and populations described by Postgate & Hunter (1961).

Enzymes. Samples of crystalline pancreatic ribonuclease (RNase) 'free from salt and protease' were obtained from the Worthington Biochemical Corp. (Freehold, New Jersey, U.S.A.) and L. Light and Co. Ltd.; a third sample of crystalline RNase was obtained from British Drug Houses, Ltd. Crystalline 'Tryptar' trypsin was obtained from The Armour Laboratories (Hampden Park, Eastbourne), crystalline muramidase and pepsin from L. Light and Co. Ltd. and non-crystalline deoxyribonuclease (DNase; activity equiv. crystalline enzyme) from the Sigma Chemical Co. Ltd. (St Louis, Missouri, U.S.A.).

Analytical methods. Cold acid-extracts of bacterial suspensions were prepared by adding the suspension (2·5 ml.) to 2 N-$HClO_4$ (0·5 ml.) at 0°. After 30 min. the acid suspensions were centrifuged and the supernatant fluids separated. Hot acid-extracts of the bacteria were prepared by resuspending the cold acid-extracted pellets in 0·5 N-$HClO_4$ (5 ml.) for 30 min. at 70°. RNA in hot acid-extracts was determined by the Bial reaction (Morse & Carter, 1949) with yeast RNA as the standard. RNase activity was determined by measurements at 260 mμ of the acid-soluble ultraviolet (u.v.) absorbing substances released from yeast RNA after exposure to the test enzyme solution for 25 min. at 25° (Joseffson & Lagerstedt, 1962). U.v.-absorption was measured in a Unicam quartz spectrophotometer, model SP. 500, with a 1 cm. light path. The fluorescence of bacterial suspensions treated with anilino-naphthalene-8-sulphonic acid (ANS) was measured in a fluorometer supplied by The Locarte Company, 24 Emperor's Gate, S.W. 7. Bacterial dry weights were determined as previously described (Strange *et al.* 1961).

RESULTS

Enhancement of cold shock by RNase

According to viability determinations by slide culture (Fig. 1) or plate counts, RNase (10–100 μg./ml.) increased the death-rate of exponential phase *Aerobacter aerogenes* washed and resuspended in dilute saline phosphate buffer at 0°, but had no immediate effect on similar unchilled suspensions at 20°. In this diluent at 0°, RNase only affected bacterial populations susceptible to the lethal effect of cold shock; stationary-phase bacteria from batch culture or steadily growing bacteria from a continuous culture (Postgate & Hunter, 1962) were not significantly affected by chilling either with or without RNase. On a given batch of organisms, the lethal effect differed according to the sample of RNase used. Mg^{2+} or filtrate from a chilled dense bacterial suspension, which protect bacteria from the lethal effect of cold shock (Strange & Dark, 1962), also protected bacteria chilled in the presence of RNase (Table 1). Sucrose (0·3 M), which also protects bacteria during chilling (Meynell, 1958; Strange & Dark, 1962), afforded slight protection in the presence of

Table 1. *Inhibition of the lethal effect of RNase on Aerobacter aerogenes in dilute saline phosphate buffer at* 0°

Washed exponential phase bacteria (about 6×10^8/ml. diluent) were diluted 1/20 into test solutions at 0°. 'Leakage products' consisted of a bacteria-free filtrate from a chilled (0°, 45 min.) *A. aerogenes* suspension (equiv. 3·8 mg. dry-wt. bacteria/ml. diluent). The diluent was dilute saline phosphate buffer (pH 6·5). Average viabilities (slide culture) from duplicate tests are shown.

Addition to diluent	Sampling time (min.) 30	60	120
	Viability (%)		
Nil	88	59	32
RNase (100 μg./ml.)	34	15	8
Mg^{2+} (5 mM)	99	99	98
RNase (100 μg./ml.) + Mg^{2+} (5 mM)	95	87	77
Leakage products	100	98	96
RNase (100 μg./ml.) + Leakage products	78	65	44
Sucrose (0·3 M)	99	97	98
RNase (100 μg./ml.) + Sucrose (0·3 M)	51	40	*

* Some bacterial lysis occurred on slide culture.

RNase (Table 1). In stronger saline phosphate buffer (0·11 M-NaCl + 0·02 M-PO_4; pH 6·5), similar results were usually obtained, but in a few experiments (four out of nineteen) with bacteria that were less than normally susceptible to cold shock, RNase did not increase the death-rate.

Tests with other enzymes. Pepsin or DNase (100 μg./ml.) had no significant effect on the death-rate of bacteria in saline phosphate buffer at 0°, whereas trypsin or muramidase (100 μg./ml.) were protective (Fig. 2). These enzymes had no immediate effect on the viability of similar bacterial suspensions at 20°.

RNA metabolism on incubation of cold shocked bacteria

Enhancement of cold shock by RNase would result from penetration of the enzyme into the bacteria followed by degradation of cellular RNA when the organisms were warmed on the recovery medium. Attempts were therefore made to confirm the entry of RNase into chilled bacteria by demonstrating an increased rate

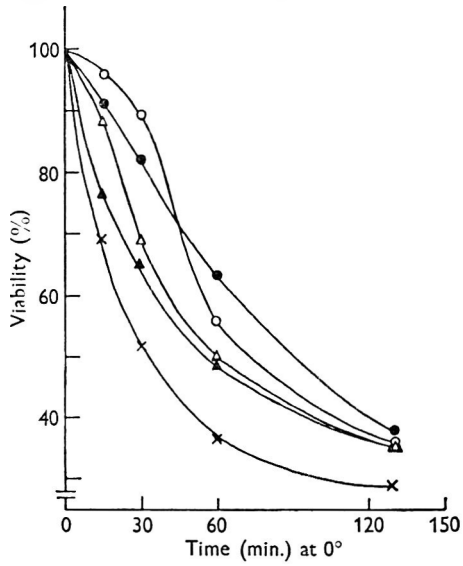

Fig. 1

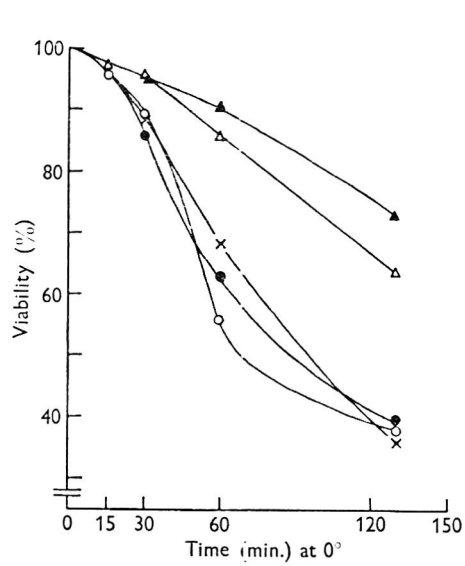

Fig. 2

Fig. 1. Survival of Aerobacter aerogenes at 0° in the presence of RNase. Washed exponential phase bacteria (about 4×10^8/ml. dilute saline phosphate buffer) were diluted 1/20 in diluents with and without RNase at 0° and 20°. Viabilities (slide culture) of bacteria at 0° in dilute saline phosphate buffer alone (○) and with 10, 25, 50 and 100 μg./ml. RNase (●, △, ▲, ×, respectively). Viabilities of suspensions at 20° were 98–100 % for 130 min.

Fig. 2. Survival of Aerobacter aerogenes at 0° in the presence of various enzymes. Viabilities of bacterial suspensions (prepared as in Fig. 1) at 0° in dilute saline phosphate buffer alone (○) and with (100 μg./ml.) DNase (●), trypsin (△), pepsin (×) and lysozyme (▲). Viabilities of similar suspensions at 20° were 98–100 % for 130 min.

of RNA degradation during subsequent incubation of the organisms. At 37°, these attempts failed because cold shock itself stimulated autodegradation of RNA: when bacteria were chilled for various times, separated from the suspensions, resuspended in fresh diluent and incubated at 37°, RNA degradation increased with the duration of chilling (Table 2). RNA degradation in a bacterial population previously chilled in the presence of RNase did not differ significantly from that in a comparable population decreased to about the same degree of viability by chilling for a longer period in buffer alone when tested at 37°. However, when cold-shocked bacterial populations of similar viabilities were subsequently incubated at 20° or 25°, RNA degradation was consistently greater when RNase had been present during chilling (Fig. 3). Little RNA degradation occurred in bacteria exposed to RNase at 20° and then subsequently incubated at 37°.

Effect of late addition of RNase. The foregoing experiments showed that cold shock rendered a proportion of a chilled bacterial population sufficiently permeable

to admit RNase. Those organisms that would not otherwise have recovered from cold shock should be sufficiently permeable to admit RNase added after chilling, though in such conditions the added RNase would only increase the rate of degradation of RNA by the population and not its death-rate. The experiments in Fig. 4 illustrate this effect: RNase added after chilling accelerated RNA degradation but not death. RNase tested at one-tenth the concentration quoted in Fig. 4 had no detectable effect.

Table 2. *RNA degradation at 37° in Aerobacter aerogenes chilled for various times at 0°*

Washed exponential-phase bacteria (equiv. 4 mg. dry wt./ml.) were diluted 1/10 in diluent at 0°. Bacteria centrifuged from chilled suspension (10 ml.) at intervals were resuspended in aerated diluent (10 ml.) at 37°. Viability (slide culture) and bacterial RNA were determined at intervals. The diluent was dilute saline phosphate buffer (pH 6·5).

	Time of incubation at 37° (min.)			Time of incubation at 37° (min.)		
Time at 0° (min.)	0	15	30	0	15	30
	% initial RNA left			Viability (%)		
0	100	—	98	99	99	99
30	99	78	64	75	32	—
60	86	64	43	33	28	25
120	87	54	28	7·7	7·4	6·9

Adsorption of RNase onto bacteria. In contrast to its behaviour in saline phosphate buffer, RNase in distilled water was more lethal to bacteria at 20° than at 0°. In a typical experiment exponential-phase *Aerobacter aerogenes* washed in distilled water were resuspended at about 4×10^7 bacteria/ml. in distilled water and in distilled water + RNase (100 μg./ml.) at 20° and 0°. Viabilities of the suspensions after 10 min. were 100 and 1·6 %, respectively, at 20°; 99·5 and 56 %, respectively, at 0°. In parallel with the lethal effect, there was considerable bacterial adsorption of enzyme (Table 3). It appeared that the uptake of enzyme was due to adsorption, not absorption, because after separating the bacteria by centrifugation and resuspending them in 0·16 M-NaCl, RNase roughly equivalent to that taken up from distilled water was released into the salt solution. Adsorption of RNase to bacteria was progressively inhibited by increasing concentrations of NaCl or KCl. The lethal effect of RNase at 20° also decreased with increasing salt concentration (Fig. 5).

Penetration of anilino-naphthalene-sulphonate (ANS) into chilled bacteria

ANS like the toluidine homologue used by Newton (1954), forms a fluorescent complex with *Aerobacter aerogenes* when the osmotic barrier is destroyed, and it has been used to test for damage to that barrier (Mathews & Sistrom, 1960; Postgate & Hunter, 1962). If bacterial permeability is affected by cold shock, the dye might penetrate chilled bacteria, causing an increase in the fluorescence of the suspension. A small but significant increase in the fluorescence of a suspension of exponential phase *A. aerogenes* containing ANS was observed on chilling (Table 4). The viability of the chilled suspension decreased more rapidly after exposure to ANS for 30 min. than that of a similar population of bacteria chilled in the absence of dye.

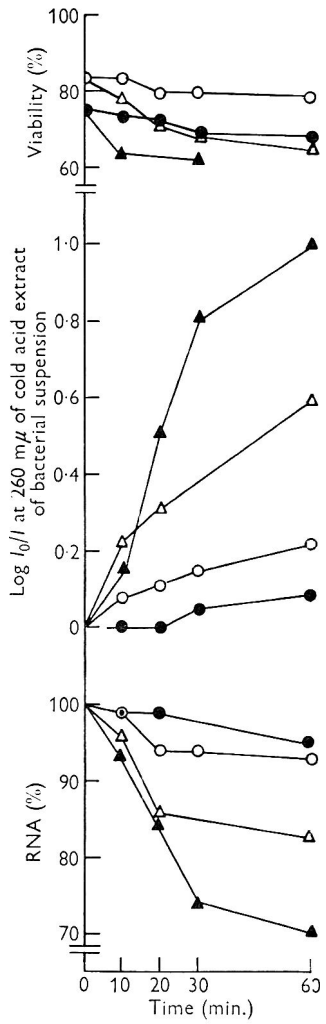

Fig. 3

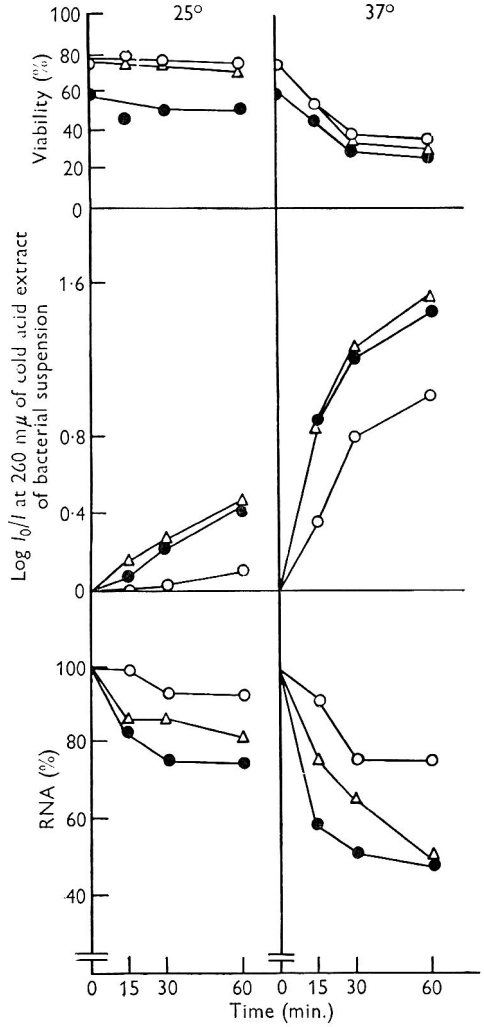

Fig. 4

Fig. 3. RNA metabolism in Aerobacter aerogenes after chilling in the presence and absence of RNase. Washed exponential phase bacteria (equiv. 6·6 mg. dry wt./ml. diluent) were diluted 1/15 in duplicate in cold (*a*) diluent + RNase (100 μg./ml.), (*b*) diluent. After chilling (*a*) 30 min. and (*b*) 60 min. the suspensions were centrifuged at 0°. After rinsing with cold diluent, one of each pair of bacterial pellets was resuspended in diluent (12 ml.) at 25°, the other at 37°. At intervals samples were taken for viability (slide culture) and analyses. Bacteria chilled in presence of RNase, open symbols; in diluent alone, closed symbols. Suspensions at 25° ○, ●; 37° △, ▲. Top, viabilities. Middle, $E^{1\,cm.}_{260m\mu}$ of cold acid-extracts. Bottom, bacterial RNA (% initial concentration). The diluent was dilute saline phosphate buffer.

Fig. 4. Bacterial RNA degradation after chilling in the presence of RNase and after chilling in the absence but incubating in the presence of RNase. Washed exponential phase bacteria (equiv. 6·09 mg. dry wt./ml. diluent) were diluted 1/10 in cold (*a*) diluent + RNase (100 μg./ml.), (*b*) diluent. After 15 min. at 0°, two samples (10 ml.) of (*a*) were centrifuged and the pellets resuspended in diluent (10 ml.) at 25° and 37°. After 45 min. at 0°, four samples (10 ml.) of (*b*) were centrifuged and the pellets resuspended in 10 ml. of (1) diluent, and (2) diluent + RNase (100 μg./ml.) at 20° and 37°. Bacteria (*a*) chilled in presence of RNase (●), (*b*) chilled without RNase and incubated (1) without RNase (○), and (2) with 100 μg./ml. RNase (△). Top, viability (slide culture); middle, $E^{1\,cm.}_{260m\mu}$ of cold acid extracts; bottom, bacteria RNA (% initial concentration). Incubation at 25°, left, at 37°, right. The diluent was dilute saline phosphate buffer.

Effect of hydrogen and hydroxyl ions on the viability of chilled bacteria

Exponential phase *Aerobacter aerogenes*, washed and resuspended in glass-distilled water, were tested at a concentration of about $4{\cdot}5 \times 10^7$ bacteria/ml. As found previously (Strange & Dark, 1962), populations of these bacteria in distilled water were much less susceptible to cold shock than similar populations in saline phosphate buffer. Low concentrations of acid added to distilled water were much more lethal

Table 3. *Adsorption of RNase by Aerobacter aerogenes*

Water-washed exponential phase bacteria (equiv. 49 mg. dry wt./ml. distilled water) were diluted 1/11 in 0–0·16 M–NaCl (1 ml.) containing RNase (100 μg.). After 10 min. at 20°, supernatant fluids from the centrifuged suspensions were separated and assayed for RNase. Bacterial pellets were resuspended in 0·16 M-NaCl (1 ml.). After 15 min. at 0°, supernatant fluids were separated and assayed for RNase released from the bacteria.

NaCl (M)	Adsorption of RNase by bacteria %	Release of RNase from bacteria in 0·16 M-NaCl %
0	95	96
0·01	51	67
0·02	32	34
0·04	14	18
0·08	10	11
0·16	10	5

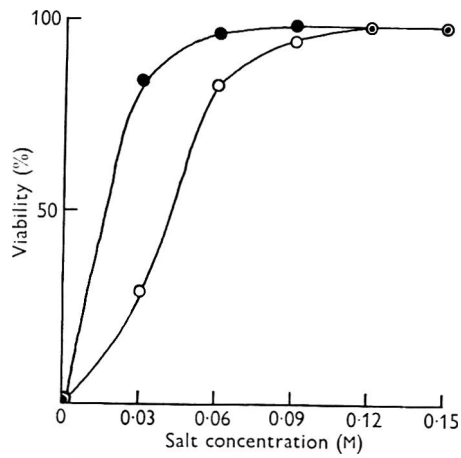

Fig. 5. Salt inhibition of the lethal effect of RNase on bacteria. Water-washed exponential phase bacteria (about 8×10^8/ml. H_2O) were diluted 1/20 in 0–0·15 M-NaCl (○) and KCl (●) containing RNase (100 μg./ml.) at 20°. Viabilities determined by slide culture after 30 min.

to bacteria at 0° than at 20°: viabilities of suspensions after 5, 10 and 20 min. in 2 mM-HCl (pH 2·7) were 78, 61 and 35 %, respectively, at 0°; 93·5, 88 and 80 %, respectively, at 20°. When 50 mM-NaCl was added to 2 mM-HCl, the lethal effect increased: viabilities of similar suspensions after times as above were 16, 4 and <1 %, respectively, at 0°; 67, 18 and 2 %, respectively, at 20°. Similarly, low

concentrations of alkali were more lethal at 0° than at 20°: viabilities of suspensions after 5, 10 and 20 min. in 2 mM-NaOH (pH 10·9) were < 1 % at 0°; 99·5, 99·5 and 98·5 %, respectively, at 20°. Even 0·5 mM-NaOH (pH 10·1) was lethal to bacteria at 0°: viabilities of suspensions in this diluent after 15 and 30 min. were 7 and 2 %, respectively, at 0°; 100 and 99 %, respectively, at 20°. Addition of 50 mM-NaCl to 2 mM-NaOH increased the lethal effect at 20° but decreased it at 0°. The effect of small concentrations of NH_4OH was similar to that of NaOH but ammonium ions (1 to 100 mM-NH_4Cl; pH 6·3–5·3) had no significant effect on the viability of chilled bacteria.

Table 4. *Penetration of anilino-naphthalene-8-sulphonate (ANS) into chilled Aerobacter aerogenes*

Washed exponential phase bacteria (about 6×10^8/ml. diluent) were diluted 1/20 at 0° and 20° in diluent and diluent + ANS (50 μM). At intervals fluorescence and viability (slide culture) of appropriate suspensions were measured. The fluorometric 'blank' was a bacterial suspension without dye at 20°; the 'standard', a similar suspension heated at 70° for 5 min., cooled and treated with ANS. The diluent was saline phosphate buffer (pH 6·5)

Sample	Time (min.)	Fluorometer reading (scale div.)	Viability %
Standard	—	14	—
Bacterial suspension + ANS at 20°	60	8·2	98
Bacterial suspension + ANS at 0°	5	8·75	86·5
	10	9·25	80·5
	15	9·30	77·5
	20	9·40	69·0
	30	9·25	63·0
	60	9·65	16·0
Bacterial suspension at 0°	15	—	63·5
	30	—	62·0
	60	—	49·0

Table 5. *Augmentation of freezing damage to Aerobacter aerogenes by RNase*

Bacteria harvested from a continuous culture (Postgate & Hunter, 1962) were washed in distilled water and resuspended (equiv. 1 mg. dry wt./ml.) in distilled water, with or without RNase (100 μg./ml.). After freezing the suspensions as drops in liquid N_2 and thawing them by dilution in saline phosphate buffer (pH 7·3) at 20°, viabilities were determined by slide culture. After 30 min. at 20°, the populations were again frozen and thawed. The viabilities of control populations with and without RNase at 20° remained unchanged (97 %) for the duration of the experiment.

Treatment of bacterial suspension	Viability (%)	
	Distilled water	Distilled water + RNase (100 μg./ml.)
Unfrozen	97	97
Frozen once	37	5
Frozen twice	19	< 1

Effect of RNase on frozen and thawed bacteria

The experiments on cold shock imply that some damage occurs to chilled bacteria which may not be lethal unless RNase is present. Postgate & Hunter (1963) examined metabolic injury resulting from freezing and thawing a bacterial population obtained

from a chemostat, and further experiments with this population showed that RNase augmented freezing damage, though the populations were insusceptible to cold shock; Table 5 illustrates these experiments. A comparable augmentation of killing on freezing and thawing in RNase solutions was observed with denser (equiv. 10 mg. dry-wt. bacteria/ml.) and sparser (equiv. 0·1 mg. dry-wt. bacteria/ml.) populations. Experiments analogous to those already described for cold-shocked populations showed that exogenous RNase augmented RNA degradation by the frozen and thawed populations.

DISCUSSION

Though Groth (1956) found that RNase inhibited growth of a special strain of *Bacillus megaterium*, and lowered the RNA content of its cultures, RNase is usually inactive towards live bacteria (Brachet, 1955). Our experiments show that cold shock makes cellular RNA accessible to extracellular RNase, and endocellular protein accessible to the dyestuff anilino-naphthalene-sulphonate and thus further support the view mentioned in the Introduction that cold shock entails damage to a permeability regulating system. The increased lethal effects of hydrogen and hydroxyl ions at low temperature are also consistent with this view. The permeability damage is not necessarily lethal, because: (i) cold shock is a progressive process (Strange & Dark, 1962), and (ii) when RNase is admitted while bacteria are 'open' it accelerates death of organisms which would otherwise have been viable, by augmenting auto-degradation of RNA. The partial reversibility of this permeability damage recalls the reversible type of freezing damage described as 'metabolic injury' (see Postgate & Hunter, 1963), which, unlike cold shock, is not restricted to exponentially growing organisms. Since exogenous RNase enhanced death and RNA degradation in frozen and thawed populations it seems likely that reversible damage to the permeability control mechanism is common to both types of stress and suggests that exponential-phase organisms differ from those growing in a chemostat only in that the latter can reverse the damage more readily.

When a bacterial population is chilled, some individuals are apparently immune and others show various degrees of susceptibility, ranging from immediate death to reversible, time-dependent damage. If susceptibility depends on the permeability status of the organisms this range of responses suggests that an exponentially growing population contains individuals of diverse permeabilities and the differences might be due to variations in permeability during a division cycle. Mitchison (1963) suggested that the permeability of a fission yeast to adenine fluctuates during the individual growth cycle. In an 'open' phase, the bacteria can admit a molecule as large as RNase (mol.wt. of monomer, 14,000; Crestfield, Stein & Moore, 1962) when chilled, yet they would have been viable were it not for the enzymic activity of this molecule. That bacteria can become permeable to large molecules and remain viable has been known since the discovery of transformation, and the rule-of-thumb procedures sometimes used to induce competence in acceptor strains (see, for example, Young & Spizizen, 1961) resemble those that might induce susceptibility to cold shock in coliform organisms. A mechanistic relationship between cold shock and transformation is possible and we attempted therefore to induce transformation of a histidineless mutant of our *Aerobacter aerogenes* by cold-shocking it in the presence of wild-type DNA. The experiment was unsuccessful, so we do not

report it in detail, but it does not exclude the relationship suggested because transformation in *A. aerogenes* has not been reported and the species may be non-transformable for quite unrelated reasons. Since DNase did not penetrate cold-shocked *A. aerogenes* it is possible, for instance, that the permeability barrier never becomes sufficiently 'open' in this species to admit molecules much larger than 14,000.

The fact that the lethal effect of RNase on *Aerobacter aerogenes* in distilled water was greater at 20° than 0° does not necessarily conflict with the conclusion that cold shock affects the permeability of susceptible organisms. Bacterial adsorption of enzyme was considerable from solution in distilled water but small from solution in saline phosphate buffer. Surface attachment of RNase is likely to facilitate its entry into the bacteria and the subsequent enzymic activity would be greater at 20° than at 0°. The progressive inhibition of both adsorption and the lethal effect of RNase at 20° by increasing concentrations of sodium or potassium chloride strongly suggests a relationship between the two phenomena.

The mechanism of the protective effects of magnesium ions, leakage products from chilled bacteria and sucrose against both cold shock and the lethal effect of RNase are not clear. These substances may affect bacterial permeability at 0°, thus preventing the entry of RNase, or they may inhibit the activity of the enzyme during subsequent incubation of the bacteria on growth medium. The latter suggestion is certainly possible in the case of magnesium which is known to stabilize isolated ribosomes (see review by McQuillen, 1962) and to diminish RNA degradation in heat stressed *Aerobacter aerogenes* (Strange & Shon, 1964). The fact that cold shock stimulates autodegradation of RNA makes it evident that interference with permeability control is not the sole traumatic consequence of cold shock, though available evidence indicates that it is the primary one.

We are indebted to Mr A. G. Ness for some analyses, Major L. H. Kent and Mr J. H. R. Slade for the preparation of anilino-naphthalene-8-sulphonate and Mr J. M. Boyle for the histidineless mutant of *Aerobacter aerogenes*. Mr J. R. Hunter performed the experiments on freezing and thawing.

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J. gen. Microbiol. (1964), 36, 405–414
Printed in Great Britain

Azelaic Acid Utilization by a Pseudomonas

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(*Received* 31 *March* 1964)

SUMMARY

A Pseudomonas isolate which utilizes azelaic acid as the sole carbon source was isolated from garden soil. Two different variants of the micro-organisms were obtained from the original culture in azelaic acid medium. One variant (s), exhibited uniform turbidity which clarified rapidly after maximal growth was obtained. The second variant (t) grew in aggregates and clumps. Both variants gave normal growth curves with a maximal stationary phase in medium with glucose or in azelate medium with high osmotic pressure. The double role of azelaic acid as a source of carbon and as a harmful agent is discussed. It was concluded that when the concentration of azelate is low enough and its action not prolonged it caused cytological disturbances which were not easy to observe (variant s). But the prolonged action of azelaic acid resulted in phenotypical changes that were partially inheritable even in its absence (variant t).

INTRODUCTION

Bacteria which utilize azelaic acid as the sole carbon source have not been described heretofore. Like other long chain dicarboxylic acids, azelaic acid may be a by-product of oxidation of fatty acids in mammalian systems (Verkade, 1938). It is also possible that azelaic acid can arise from alkanes. Alkanes are known to serve as a source of six and seven carbon dioic acids (Colla & Treccani, 1960) or ten and more carbon dioic acids (Kester & Foster, 1963). The capacity to oxidize azelaic acid by bacteria is interesting from the point of view of the carbon cycle in nature, and also because of the fact that this nine carbon compound is one of the precursors of biotin in the growth of some micro-organisms (Wright, Cresson & Driscoll, 1955). The aim of the present work was to isolate bacteria able to utilize azelaic acid and to establish the best conditions for investigations of azelaic acid degradation.

METHODS

Organisms. The original culture of *Pseudomonas* sp. strain w was isolated in our laboratory from garden soil by the enrichment culture technique. Organisms were maintained on agar slopes of the following composition (%, w/v): $(NH_4)_2SO_4$, 0·1; K_2HPO_4, 0·05; $MgSO_4.7H_2O$, 0·01; $FeSO_4.7H_2O$, 0·002; NaCl, 0·01; azelaic acid, 0·1, 0·2, or 0·3; agar, 1·2; distilled water. This and all subsequent media were adjusted to pH 7·0 with dilute NaOH before final sterilization. Isolate w was sub-cultured every week.

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Media. Azelaic acid liquid medium unbuffered: the same composition as azelaic acid solid medium. Azelaic acid liquid medium buffered: prepared with phosphate buffer: KH_2PO_4, 4·0 g. and K_2HPO_4, 13·6 g. per 1000 ml. instead of distilled water. Special media for identification purposes (%, w/v): carbohydrate media: synthetic liquid medium with Durham tubes: $NH_4H_2PO_4$, 0·1; KCl, 0·02; $MgSO_4.7H_2O$, 0·02; carbohydrate, 0·5; distilled water. Peptone-carbohydrate semisolid medium of Hugh & Leifson (1953): peptone, 0·2; NaCl, 0·5; K_2HPO_4, 0·3; agar, 0·3; bromthymol blue, 0·003; carbohydrate, 1·0; distilled water. King, Ward & Raney (1954) medium A for enhancement of pyocyanin and pyorubrin: bacto peptone, 2·0; bacto agar, 1·5; glycerol, 1·0; K_2SO_4 (anhydr.), 1·0; $MgCl_2$ (anhydr.), 0·14; distilled water. King *et al.* medium B for enhancement of fluorescin: proteose peptone, 2·0; bacto agar, 1·5; glycerol, 1·0; K_2HPO_4 (anhydr.), 0·15; $MgSO_4.7H_2O$, 0·15; distilled water.

Isolation and maintenance of different variants of micro-organisms. Variants S and T (transition) were obtained by subcultivation from growth of isolate W in azelaic acid liquid medium, to azelaic acid agar plates. Both variants were un-stable on azelate medium after several transfers changing partially to the opposite variant. The variant S was purified several times by single colony selection and has been maintained on nutrient agar slopes. Variant T was more stable in azelate medium and in such a medium has been cultivated.

Morphological and cultural characteristics. Organisms were stained by Gram's method and for flagella by Leifson's method after incubation for 1 day at 22° or 30° on nutrient agar. Their size in hanging drop, on nutrient agar, and in azelate culture during 14 days of incubation was measured. Observations were made on the first subculture after isolation or re-isolation.

Physiological tests. Most of the physiological tests were made according to procedures given by the *Manual of Microbiological Methods* (1957). A standard inoculum for all tests was one loopful of 1-day nutrient broth culture. All media were incubated at 22°. The ability of strain W to grow in nutrient broth at 42°, 37°, 30°, 25° 10°, 6° and 1° was noted, the incubation periods extending to 1 month at the lower temperatures.

The medium of Hugh & Leifson (1953) in sealed and unsealed tubes was used to distinguish oxidation from fermentation of carbohydrates.

The diffusible pigment production was examined with the media of King *et al.* (1954); oxidase activity was checked according to the method of Gaby & Hadley (1957).

Growth measurements. Growth of shaken cultures incubated at 30° was followed turbidimetrically by using a Bausch & Lomb 340 colorimeter. The correlation between percentage of extinction (wavelength 660 mμ) and dry weight of washed organisms of strain W was determined. A standard inoculum for all growth tests was 0·2% (v/v) of 1-day culture.

Changes in pH value of cultures. Readings of the pH value of the samples were made with a Beckman Model G pH meter.

Determination of acids. A partition chromatographic method for the determination of the total amount of acids in the supernatant solutions of cultures after bacterial centrifugation was used. The method used was essentially that of Isherwood (1946); silica gel was prepared from Mallinckrodt silicic acid. Acids after

different periods of bacterial growth were isolated and titrated with dilute standard NaOH. Total amount of acids in equivalents of azelaic acid is given. The average error of estimations was 2%.

RESULTS

Morphological and cultural characteristics

Cell morphology on nutrient agar. Pseudomonas strain W showing monotrichous rods, $0{\cdot}6 \times 1{\cdot}8\ \mu$, occurring singly and in pairs. Characteristic arrangement of organisms side by side was observed. Gram negative.

Variant S: like strain W.

Variant T: monotrichous rods, $0{\cdot}6 \times 1{\cdot}6$ to $8{\cdot}0\ \mu$, occurring singly, in pairs and in very tight aggregates.

Cell morphology in azelaic acid medium. Strain W: exact observations lacking.

Variant S: in shaken and unshaken 1-day cultures a tendency for longer rods to develop (length, $3{\cdot}2\ \mu$) was observed. In 2-day cultures single organisms (length, $1{\cdot}8\ \mu$) and small amount of aggregates were noted. In older cultures the degree of aggregates fluctuated with the time of incubation.

Variant T: in 1–2-day shaken cultures a great variety of forms was regularly seen: short rods (length, $0{\cdot}8\ \mu$), curved filaments (length, $16\ \mu$) and hundreds of small rods in very tight aggregates. In older cultures fewer filamentous forms were noted. In unshaken cultures length of filaments varied around $65\ \mu$; filaments were seen dividing into coccoidal forms; a large number of aggregates was always seen.

In buffered azelaic acid medium both variants S and T grew alike. In shaken culture the organisms were single and after the second day of incubation short organisms only were observed (S, length, $0{\cdot}6$–$1{\cdot}6\ \mu$; T, length, $0{\cdot}6$–$1{\cdot}6\ \mu$ and exceptionally up to $4{\cdot}8\ \mu$). In unshaken S and T cultures during the first 4 days of incubation single rods and chains were observed. After 5 days there were also curved filaments (length, $50\ \mu$) without transverse walls. In older cultures single organisms, swollen organisms, chains, curved filaments, filaments with granulated plasma, and rarely aggregates were seen.

Morphology of organisms grown in azelaic acid medium supplemented with 5·8% *NaCl.* Organisms of both variants S and T in medium with high osmotic pressure grew like organisms in buffered medium. Round swollen forms ('spheroplasts') were also seen.

Colonial morphology on nutrient agar. Strain W: 48 hr colonies were circular, about 2 mm. in diameter. On further incubation, these colonies became larger, about 5 mm., elevated and with filamentous margins. The surface was smooth, glistening, the centre of the colonies yellow, the periphery transparent.

Variant S: like strain W, only the margin of colonies was entire or undulate.

Variant T: circular yellow colonies with a curled appearance. On prolonged incubation, smooth and transparent outgrowths projected from the margins.

Colonial morphology on azelate agar. Strain W: 48 hr colonies were circular, about 2 mm. in diameter. After incubation for 14 days, colonies became about 8 mm. in diameter, raised and with entire margin; on further incubation the margin became arborescent. The surface was smooth, glistening, gold-yellow. Some colonies developed smooth secondary colonies on their surfaces.

Variant S: like strain W, but without the smooth secondary colonies. In older colonies concentric rings with different transparence were also observed.

Variant T: as on nutrient agar.

Appearance in liquid media. Strain W: in broth, broth + yeast extract and in peptone water, strain W produced strong turbidity, sediment and pellicle. Formation of the pellicle in azelate cultures was variable. Growth in shaken azelate cultures was at first uniform. After a few transfers in some flasks smaller and bigger grains became visible. Aeration by bubbling stimulated the production of clumps. From these cultures, variants S and T were re-isolated. Near the end of the exponential phase of well growing cultures, the supernatant fluid became yellow. A depression of the surface tension paralleled the growth of the bacteria.

Variant S: uniform heavy turbidity in unshaken and in shaken azelate cultures, with yellow colour of supernatant fluid near the end of the growth.

Variant T: turbidity, clumps and pellicle in unshaken azelate cultures; turbidity, smaller and larger grains in shaken culture. As in variant S, the supernatant fluid of well-developed cultures became yellow.

Physiological characteristics of strain W

Gelatin liquefaction—negative.

Nutrient gelatin liquefaction—negative.

Action on litmus milk—alkalization without litmus reduction.

Reduction of nitrate to nitrite—nitrate reduced in 24 hr to nitrite, followed by nitrite reduction to ammonia and nitrogen.

Ammonia production—positive.

Hydrogen sulphide formation—slightly positive.

Indole formation—positive after 24 hr incubation at 30°.

Production of acid from carbohydrate in synthetic media:

(*a*) Acid from: D + xylose (1·5*); D + glucose (3·0*);
D + galactose (3·0*); D − levulose (2·0*);
D + maltose (3·0*).

(*b*) Small amount of acid from: D + mannose (1·0*); D + lactose (1·0*).
(*decrease of pH value during 10 days of incubation.)

(*c*) No acid from: L − xylose; L + arabinose; D − arabinose;
L sorbose; sucrose; D + melibiose;
D + trehalose; D + raffinose; L + rhamnose.

No gas was produced from any above-mentioned sugars in synthetic media or in media with the addition of peptone. No acid was produced in sealed tubes in the medium of Hugh & Leifson (1953).

Starch hydrolysis—negative.

Growth in Koser's citrate medium—positive.

Voges-Proskauer and methyl red tests—negative.

Greenish yellow pigment on the medium for enhancement of fluorescin (King *et al.* 1954).

Oxidase production—positive.

Temperature range of growth 6–35°; no growth at 37°.

Azelaic acid bacteria strain w is considered to be a *Pseudomonas* sp. because it: (1) is Gram-negative and motile with a single polar flagellum; (2) produces a greenish yellow water-soluble pigment; (3) produces acids oxidatively from carbohydrates.

Morphological and physiological characteristics of this organism suggest that the isolated strain belongs to a species never before described in the literature.

Growth of Pseudomonas strain w *in defined medium with azelaic acid*

To establish the growth curve of the Pseudomonas strain w several nephelometer readings of the shaken culture growing with 0·3 % azelaic acid were made. Simultaneously samples from the same culture were centrifuged to obtain the dry weight of organisms in the sample. Results from this experiment show rapid growth of micro-organisms and unusually rapid decrease of cultural turbidity (Fig. 1). During this growth slightly acid (pH 6·7) medium became strongly alkaline (pH 9·4).

The tested strain never grew similarly in the subsequent experiments, in the same or in other growth conditions (different temperature, different amount or age of the inoculum). When different concentrations of azelaic acid were used (0·1, 0·2, 0·3 %), repeatable results were obtained solely with 0·1 % azelaic acid (Fig. 2).

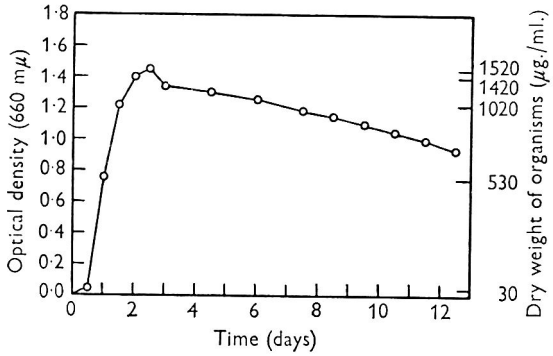

Fig. 1

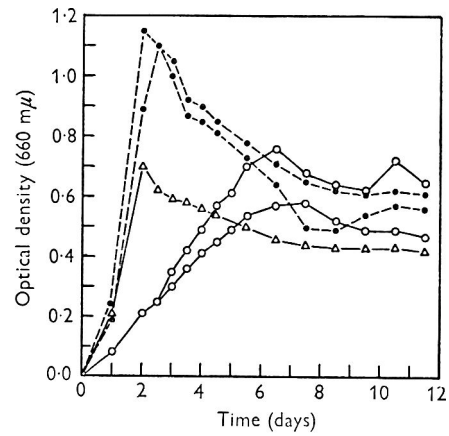

Fig. 2

Fig. 1. Growth curve for strain w after ten transfers through azelate medium. Growth in medium with 0·3 % of azelaic acid.

Fig. 2. Growth curve for strain w after twenty transfers through azelate medium. Growth in medium with different concentrations of azelaic acid: ○——○, 0·3 % azelaic acid; ● – – ●, 0·2 % azelaic acid; △——△, 0·1 % azelaic acid.

Microscopical observations of growing cultures revealed a large amount of aggregates of cells in flasks with low turbidity. Subculturing of strain w on plates gave two types of colonies from which variant s and variant r were re-isolated. Growth of both variants was determined in buffered and unbuffered medium containing 0·1 % azelaic acid. The aggregates of variant r strongly influenced the nephelometer readings in unbuffered medium. Addition of the mono- and dibasic potassium phosphates in amounts mentioned in 'Methods' gave very rapid growth. The slope of the growth curve after maximal growth was not so sharp as usual for both variants (Fig. 3).

Small changes of pH value during the first 2 days of incubation of unbuffered cultures (pH 6·7–7·3) did not indicate that the alkalinity was the factor inhibiting growth.

Total acid utilization in culture medium was approximately the same for both strains (Fig. 3).

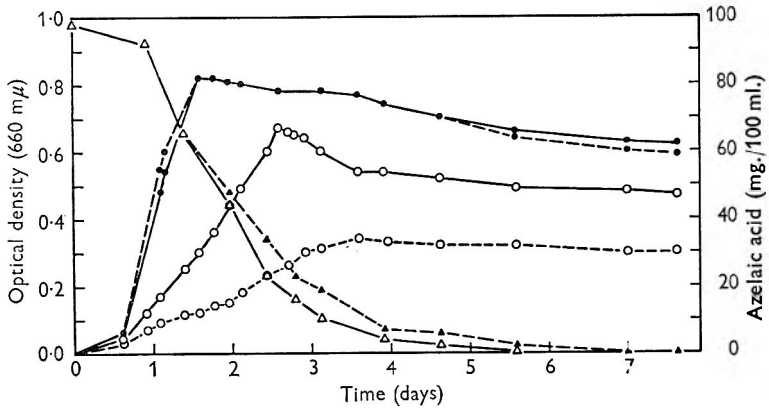

Fig. 3. Growth and azelaic acid utilization by variants S and T in medium with 0·1 % of azelaic acid. ○——○, Growth of variant S; ○--○, growth of variant T; ●——●, growth of variant S in buffered medium; ●--●, growth of variant T in buffered medium; △——△, azelaic acid uptake by variant S; ▲--▲, azelaic acid uptake by variant T.

Some characteristics of S *and* T *variants of azelaic acid bacteria*

From the above-described experiments, the question arose as to whether or not variant T retains its special features after several cell divisions in buffered azelate medium. At the same time, the data indicated that the filtrate of the old culture of the tested micro-organisms contains some lytic enzymes which dissolve damaged bacterial cell walls.

Two 25 ml. samples of the culture of each variant at the maximal stationary growth phase in buffered medium were centrifuged and washed several times. The organisms were then placed in 45 ml. Ringer's solution in Erlenmeyer flasks and the mixture made homogeneous by mechanical shaking. After shaking for 4 hr, 5 ml. water was added to one flask and 5 ml. sterile filtrate of the old culture of the same bacteria was added to the second flask. Measurements of extinction of the fluid were made until a uniform suspension of organisms was achieved.

Overall homogenization of variants S and T took 76 and 112 hr respectively (Fig. 4). The addition of the filtrate of old culture strongly influenced the attainment of homogeneity, the rate of which was enhanced by the filtrate and was the same for both variants. If azelaic acid is the cause of the partially inheritable changes of outside layers of bacteria, then perhaps when unprotected by the normal cell walls, micro-organisms form aggregates, undergo lysis, and die rapidly in hypotonic solution. For this reason, both strains were inoculated into the medium with high osmotic pressure.

Growth in the medium with 0·1 % azelaic acid and 5·8 % NaCl started after incubation for 48 hr and continued slowly during the next few days. The growth

curve of both strains was 'normal' (Fig. 5) and turbidity was homogeneous. Cultures without NaCl served as controls and showed that both variants in the medium with azelaic acid retained their characteristics.

No abnormal damaging of microbial cells and no differences between variants s and t in defined medium with glucose were observed. Maximal turbidity of cultures with glucose (added in an amount equivalent to the amount of carbon supplied by 0·1 % azelaic acid) was stable, but one half that in azelaic acid culture of variant s (Fig. 6). The lower final acidity of the culture with glucose (pH 4·1–4·3) could be the growth-inhibiting agent.

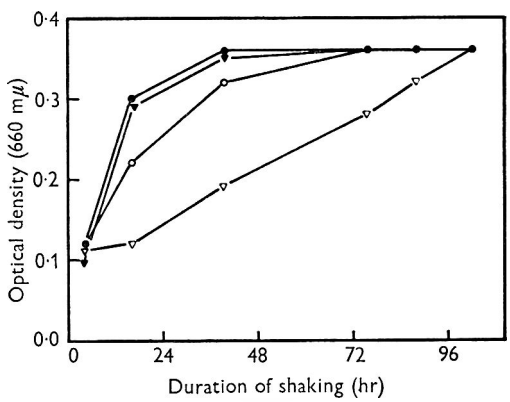

Fig. 4

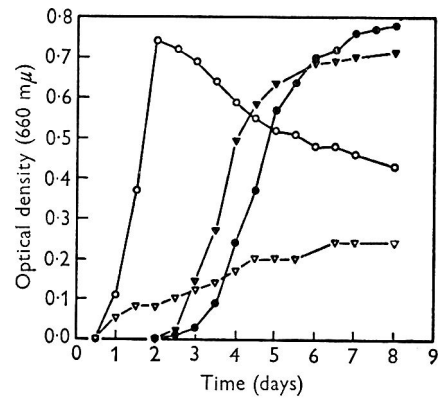

Fig. 5

Fig. 4. Homogenization by mechanical shaking of variants s and t in Ringer's solution with and without sterile filtrate of an old culture. ○——○, Variant s in Ringer's solution; ●——●, variant s in Ringer's solution with filtrate of an old culture; ▽——▽, variant t in Ringer's solution; ▼——▼, variant t in Ringer's solution with filtrate of an old culture.

Fig. 5. Growth curves for variants s and t in the medium with 0·1 % of azelaic acid and different concentrations of NaCl. ○——○, growth of variant s in medium with 0·01 % NaCl; ●——●, growth of variant s in medium with 5·8 % NaCl; ▽——▽, growth of variant t in medium with 0·01 % NaCl; ▼——▼, growth of variant t in medium with 5·8 % NaCl.

Effect of cultures of azelate-grown pseudomonads on the growth of Streptococcus lactis

In a preliminary experiment, it was observed that autoclaved supernatant fluid from old cultures of azelate-grown pseudomonads had a growth-stimulating effect on *Streptococcus lactis* under conditions where lipoic acid was the limiting factor for growth. This suggested the possibility that azelaic acid or a metabolite of it served as a precursor of lipoic acid. To test this hypothesis a bioassay of supernatant fluids from cultures of azelate-grown pseudomonads which had been incubated with 0·1 % azelaic acid or with glucose, was made. In both cultures of azelate-grown pseudomonads the total amount of carbon was the same before inoculation. Samples were taken with sterile precautions after different incubation times, centrifuged and the clear supernatant solutions autoclaved for 15 min. at 120°. At the same time the extinction and the pH value were taken as a measure of growth. After 5 days, when the last sample for the bioassay was prepared, the remaining amounts of the cultures were autoclaved at 120° for 3 hr with sulphuric acid at pH 1·5, and the hydrolysates

neutralized and used for additional tests. The assay procedure was that described by Reed, DeBusk, Johnston & Getzendaner (1951). Nine ml. of assay medium was supplemented with 1 ml. of (1) supernatant solutions, (2) hydrolysates of culture, (3) sodium acetate solutions. The growth response of *Streptococcus lactis* 9936 group N was tested as a function of the various supplements. A stimulating effect of the supernatant fluid from the azelate-grown pseudomonads culture was observed, which increased with age of the culture. The supernatant solutions from the culture of bacteria utilizing glucose were inactive (Fig. 7). The hydrolysates from both cultures were active and the extinctions of the *S. lactis* cultures were 0·25 and 0·21, respectively.

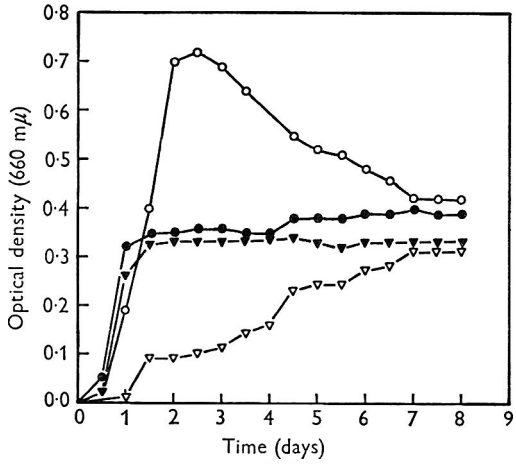

Fig. 6

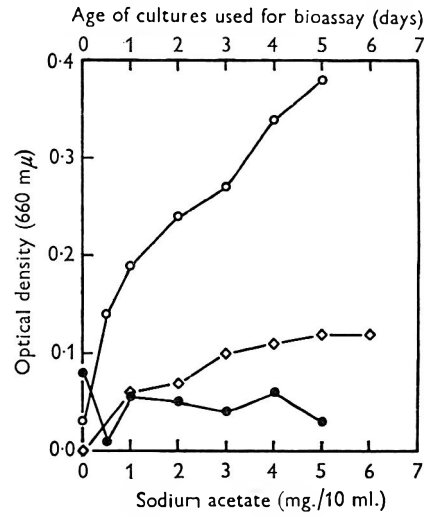

Fig. 7

Fig. 6. Growth curves for variants S and T in medium with 0·1 % of azelaic acid and in medium with 0·1436 % of glucose. ○——○, Growth of variant S in medium with azelaic acid; ●——●, growth of variant S in medium with glucose; ▽——▽, growth of variant T in medium with azelaic acid; ▼——▼, growth of variant T in medium with glucose.

Fig. 7. Growth of Streptococcus lactis after 18 hr incubation in Guirard's medium supplemented with sodium acetate or the supernatant from azelate bacteria cultures. ◇——◇, Medium with Na acetate; ○——○, medium with supernatant from culture with azelaic acid; ●——●, medium with supernatant from culture with glucose.

This insignificant difference in the growth-stimulating activity of acid-hydrolysed cultures does not prove the hypothesis that azelaic acid has a special role in the biosynthesis of the growth factor determined. On the basis of this experiment one can only suspect the greater permeability of the cell walls (perhaps in damaged bacteria) of the micro-organisms growing at the expense of azelaic acid.

DISCUSSION

The measurement of the extinction of cultures of azelate-grown pseudomonads revealed such a great irregularity of growth that it was decided to examine more carefully the morphology of the bacteria concerned, especially those from media containing azelaic acid. The smooth secondary colonies situated on the primary

colonies and the outgrowths of strain w resembled the variations in *Lactobacillus casei* described by de Klerk & Coetzee (1962*a*, *b*). However, neither of the re-isolates of azelate bacteria (s and t variants) could be identified as a smooth or a rough form. Also Tween 80, a smooth-colony-inducing agent, added to the culture of variant t did not change the features of that form. On the other hand, the t variant of azelate-grown bacteria and the L-type transition form described by Klieneberger-Nobel (1960) have some characteristics in common: the dark centre and a lighter periphery of the colony and growth in liquid media in the form of clumps.

The observations on the release from the pseudomonads growing on azelate of a growth factor for *Streptococcus lactis*, and the release of the pigment indicated that some factor damaged the pseudomonads such that the cell walls become more permeable. Damaged walls may also be more easily digested by lytic enzymes. The observations on the formation of bacterial aggregates and the decrease of the surface tension from the beginning of growth were indications that azelaic acid itself was harmful for microbes which probably release fatty acids from their envelope. The cell-wall autolytic activity increasing toward the end of the log. phase of *S. faecalis* is described by Strampp, Conover & Shockman (1963). Weidel, Frank & Leutgeb (1963) used sodium dodecylsulphate to suppress the damaging effect of lytic enzymes during preparation of the wall of some Gram-negative bacteria. This compound was inactive in our experiments. Also uranyl nitrate, a protecting agent against lysis by anionic and cationic detergents (Razin & Argaman, 1963), was without effect on the growth curve of azelate-grown pseudomonads. It remains to be tested whether or not high concentrations of salts in the culture medium will protect damaged bacteria against bursting in hypotonic solution. Both buffered and NaCl media prevented visible damage of the pseudomonads when aggregated. Both media induced the bacteria to produce aberrant forms in unshaken culture. The data presented in this work are not sufficient to explain changes in the walls and death of tested bacteria under the influence of azelaic acid and eventually under the influence of some metabolic products. For such an explanation, additional research would have to be done, taking into consideration the effect of inorganic cations which, when present in the medium in large amounts, can act by decreasing the negative charge at the cell surface (Voss, 1963). It seems, however, that the results described above prove the double role of azelaic acid: (1) as a source of carbon, (2) as a harmful agent. When the concentration of azelate is low enough and its action not prolonged, it causes cell disturbances not easy to observe (variant s). But the prolonged action of azelaic acid results in phenotypical changes, partially inheritable even in its absence (variant t).

Pseudomonas sp. strain w has been deposited with the American Type Culture Collection, Washington, D.C., U.S.A.

We wish to thank Dr H. H. Seeley, Jr. for the supply of a strain of *Streptococcus lactis* 9936 group N used in our research. This investigation was supported by Public Health Service Research Grant No. 4138 from the National Heart Institute.

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J. gen. Microbiol. (1964), 36, 415–422
Printed in Great Britain

Nitrogen Fixation by Myxophyceae from Marine Environments

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SUMMARY

Two blue-green algae from marine environments, *Calothrix scopulorum* and *Nostoc entophytum*, vigorously fixed ^{15}N-labelled nitrogen in artificial sea-water free from combined nitrogen and, rather faster, in natural sea-water. A proportion of the nitrogen fixed was liberated into the medium. After a 7-day exposure period the extracellular nitrogen had a lower enrichment with ^{15}N than the intracellular nitrogen. Fixation rates were not markedly affected by large variations in salinity of the medium. Calothrix was more resistant to changes in salinity than was Nostoc. Fixation in sea-water collected at various seasons was highest in January samples and lowest in July samples; additions of phosphate and of trace elements to the latter stimulated fixation. Ammonium-nitrogen did not inhibit fixation completely. It is probable that these species fix some nitrogen in Nature.

INTRODUCTION

Stewart (1962, 1963) reported on the basis of long-term growth analyses that the two blue-green algae *Calothrix scopulorum* and *Nostoc entophytum* isolated from marine environments fix atmospheric nitrogen and liberate a proportion of the nitrogen fixed into the medium. This extracellular nitrogen did not appear to be due to cell autolysis. It was felt desirable to confirm these data using the more sensitive and more convincing isotopic method employing ^{15}N, and to determine whether these processes are likely to occur in Nature. One factor which may be important is the nutritional status of the environment for there is evidence that this fluctuates sufficiently at various seasons of the year to affect the metabolism of many if not all marine algae (see, for example, Black & Dewar, 1949). Combined nitrogen in the environment may also inhibit or completely suppress fixation and it is important to determine whether it is ever likely to do so in marine habitats. As the algae under investigation are abundant on the upper littoral and supra-littoral fringe of the seashore where large variations in salinity occur the effect of salinity on growth and fixation should be investigated. Finally from the point of view of further studies on these organisms the optimum conditions for growth and nitrogen fixation should be determined.

METHODS

Organisms. The algal strains employed were those used previously (Stewart, 1962, 1963, 1964) and were available in pure culture. Inocula were prepared by shaking algae in the exponential growth phase with glass beads until a homogeneous suspension was obtained (Fogg, 1942).

Sea-water. All samples used were collected from the shore at Gibraltar Point Nature Reserve, Lincolnshire. They were filtered through a Millipore HA membrane filter immediately upon collection and stored in darkness at 5° until required.

Isotopic tests. Algae which had been grown routinely in nitrogen-free medium (Stewart, 1962), or in natural sea-water, were inoculated into fresh medium and exposed to an atmosphere enriched with ^{15}N. The gas mixture was prepared by the method of Bond & Scott (1955). Gaseous nitrogen containing excess ^{15}N was liberated by the reaction between sodium hypobromite and ^{15}N-enriched ammonium chloride in a nitrometer. Oxygen, carbon dioxide, and argon were obtained from cylinders (British Oxygen Company) to give a final mixture (v/v) of 70 % A, 15 % N_2, 10 % O_2 and 5 % CO_2. This was allowed to equilibrate for at least 2 hr before being introduced into 40 ml. capacity test-tubes or conical flasks which contained the algal samples each generally of 1·0–2·0 mg. nitrogen. The containers were attached to a manifold for gassing and the system was evacuated and flushed several times with argon before introducing the gas mixture until a pressure of 1 atmosphere was obtained. The containers were then shaken for several minutes, or continuously, to allow the medium to become saturated with the gas. The degree of enrichment of the gaseous nitrogen with ^{15}N and the culture conditions during exposure varied with the experiment and are presented with the results. After exposure algae and media were separated through a Millipore HA membrane filter. Each sample was subjected to micro-Kjeldahl analysis using the catalyst mixture of Jacob (1959). Distillation was carried out in a Markham still and ammonia determined by nesslerization (Burris & Wilson, 1957). ^{15}N data were obtained by mass spectrometer (Associated Electrical Industries, Type MS 3) assay.

Long-term experiments. The effect of salinity concentration, and of ammonium-nitrogen, on fixation were investigated by inoculating the algae into 25 ml. samples of medium in 100 ml. conical flasks stoppered with cotton wool, and shaking continuously during the experimental period at 80 oscillations/min. Algae and media were separated by filtration through weighed Whatman no. 50 filter-paper, washed to remove media, and algal dry weights obtained by drying overnight at 95°. Total nitrogen was estimated as described above.

(1) *Salinity experiments.* The basal media employed are presented in Table 1. The desired salinities were obtained by diluting or concentrating solution 1, solution 2 being maintained always at 1·0 ml./l. of final medium. Before inoculation the algae were transferred through six changes of distilled water to minimize carry-over of the original medium, algae and media being separated by centrifugation at each transfer. Aliquots of inoculum were added to 15 flasks at each of the following salinity levels: 0·1, 1·0, 2·0, 3·5, 4·5, 6·0 and 10·6 % (w/v), and the contents of triplicate flasks at each treatment harvested every 3 days over a 15-day period and dry-weight data obtained as above.

(2) *Combined nitrogen experiments.* The effect of combined nitrogen on fixation was studied using a modification of the method of Stewart & Bond (1961) for higher plants. Triplicate flasks, containing 50 mg./l. of ^{15}N-enriched ammonium chloride, were inoculated with algae and placed under alternating periods of 18 hr light (mean intensity 390 foot-candles) and 6 hr darkness. The salinity of the medium was 3·5 % (w/v) (above), mean temperature 26°, and initial pH 8·0. During the

14-day experimental period pH levels, salinity concentrations, and ammonium-nitrogen concentrations were maintained near to normal by the daily addition, when necessary, of sodium hydroxide, distilled water or ^{15}N-enriched ammonium chloride.

Table 1. *Media used in preparation of various salinity levels*

Solution 1		Solution 2	
NaCl	24·80 g.	K_2HPO_4	0·25 g.
$MgCl_2.6H_2O$	10·89 g.	Fe citrate	0·01 g.
$CaCl_2$	1·19 g.	Citric acid	0·01 g.
K_2SO_4	0·95 g.	Fe (as chloride)	0·40 mg.
$Na_2SO_4.10\ H_2O$	7·30 g.	Mn (as chloride)	0·10 mg.
Distilled water	999 ml.	Mo (as Na salt)	0·10 mg.
		B (as boric acid)	0·10 mg.
		Cu (as sulphate)	0·01 mg.
		Zn (as sulphate)	0·01 mg.
		Distilled water	1·0 ml.

RESULTS

Tests for fixation of elemental nitrogen and for liberation of fixed nitrogen into the medium. In initial tests the algae were inoculated into 10 ml. samples of nitrogen-free medium or April 1963 sea-water and 48 hr later exposed to ^{15}N for 7 days. The data (Table 2) show that Calothrix and Nostoc assimilate elemental nitrogen in both media, fixation in natural sea-water being greater.

Table 2. *Assimilation of ^{15}N by Calothrix and Nostoc cultured in nitrogen-free medium and in natural sea-water*

Alga	Medium	Sample	Atom % excess ^{15}N in alga	
Calothrix	Artificial N-free	1	7·558	(8·270)
		2	9·327	
		3	7·924	
	Natural sea water	1	12·105	(11·934)
		2	11·762	
Nostoc	Artificial N-free	1	8·744	(8·589)
		2	8·613	
		3	8·410	
	Natural sea water	1	12·312	(12·379)
		2	12·446	

Period of exposure = 7 days; labelling of gaseous N = 17 atom % ^{15}N; volume of medium = 10 ml.; volume of gas = 30 ml.; light intensity = 350 foot-candles; mean temperature = 26°; mean ^{15}N content of inocula = 0·372 atom %; figures in parentheses are means.

Data on growth in nitrogen-free medium at various salinity levels (Fig. 1) indicate that nitrogen fixation occurs over wide salinity ranges. There is no well-defined optimum salinity level for either species. Growth of Calothrix is not significantly different (P = 0·05 level) at salinities of 0·1–4·5 % (w/v), although there is some evidence of slightly better growth at 1·0–3·5 % (w/v). At 6·0 % (w/v) growth is significantly poorer than at lower salinities. At 10·6 % (w/v) no increase in dry weight occurred during the 15-day period, although such cultures resume normal growth if the salinity is reduced to 6·0 % (w/v) or lower. The Nostoc growth rates are similar at 1·0–3·5 % (w/v), but again there is evidence of slightly better growth near to 2·0 % (w/v). Growth at 4·5 % (w/v) is significantly poorer than at the lower

levels. Little growth occurred at 6·0 % (w/v), while at 10·6 % (w/v) no increase in dry weight occurred. Cultures exposed to the latter treatment fail to multiply on reduction of the salinity level to 4·5 % (w/v) or lower, indicating that exposure to such a high salinity for such a period is toxic to the algae.

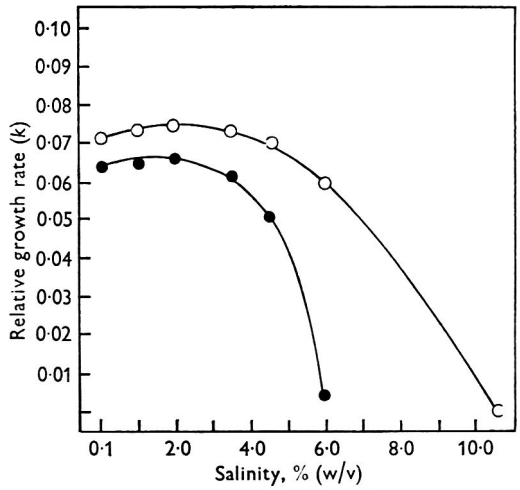

Fig. 1. The growth rates of Calothrix (○——○) and Nostoc (●——●) in solutions of differing salinity.

Table 3. *Fixation and liberation of* ^{15}N *by Calothrix and Nostoc*

Alga	Medium	Sample	Atom % excess ^{15}N in alga	Atom % excess ^{15}N in medium	Atom % excess ^{15}N in medium as % of atom % excess ^{15}N in alga
Calothrix	Artificial N-free	1	0·352	0·103	29
		2	0·401 (0·408)	0·310 (0·187)	77 (46)
		3	0·470	0·148	32
	Natural sea-water	1	0·794	0·321	40
		2	0·826 (0·861)	0·288 (0·351)	35 (40)
		3	0·963	0·443	46
Nostoc	Artificial N-free	1	0·356	0·135	38
		2	0·462 (0·438)	0·194 (0·181)	42 (41)
		3	0·495	0·213	43
	Natural sea-water	1	0·514	0·303	59
		2	0·414 (0·437)	0·158 (0·203)	38 (46)
		3	0·383	0·158	41

Period of exposure = 7 days; labelling of gaseous N = 3 atom % ^{15}N; volume of medium = 25 ml.; volume of gas = 15 ml.; light intensity = 350 foot-candles; mean temperature = 24°; mean ^{15}N content of inocula = 0·370 atom %; figures in parentheses are means.

Once fixation was established data on the liberation of extracellular nitrogen were obtained. The algae were transferred into 25 ml. samples of fresh medium and immediately exposed to ^{15}N, after which they were gently shaken during the exposure period. The data (Table 3) show that fixed nitrogen is liberated into nitrogen-free medium and into sea-water. The extracellular nitrogen has a lower labelling with ^{15}N than the intracellular nitrogen.

Assimilation of ^{15}N in sea-water samples from different seasons. Tests which employ long exposure periods, while necessary to determine whether fixation occurs, have the disadvantage in that during exposure the medium may change as a result of algal metabolism. This can be overcome to some extent by inoculating a small algal sample into a relatively large volume of sea-water and employing a short exposure period. This method was employed in tests for fixation by algae inoculated into sea-waters collected in July 1962, October 1962, January 1963 and April 1963. The algae were transferred every 24 hr, employing the methods detailed earlier, into fresh 25 ml. samples of these sea-waters during the 7 days before exposure to ^{15}N. The data (Table 4) show that both algae assimilate gaseous nitrogen in all sea-

Table 4. *Assimilation of ^{15}N by Calothrix and Nostoc when grown in sea-waters collected at various seasons of the year*

		Atom % excess ^{15}N in alga	
Sea-water	Sample	Calothrix	Nostoc
January 1963	1	0·254	0·203
	2	0·233 (0·244)	0·193 (0·198)
April 1963	1	0·189	0·182
	2	0·207 (0·198)	0·186 (0·184)
July 1962	1	0·184	0·165
	2	0·176 (0·180)	0·152 (0·159)
October 1962	1	0·211	0·192
	2	0·202 (0·207)	0·186 (0·189)

Period of exposure = 3 hr; labelling of gaseous N = 30 atom % ^{15}N; volume of medium = 25 ml.; volume of gas = 15 ml.; light intensity = 600 foot-candles; mean temperature = 25°; mean ^{15}N content of inocula = 0·370 atom %; figures in parentheses are means.

Table 5. *The effect of phosphate and of trace elements on nitrogen fixation by Calothrix and Nostoc in natural sea-water*

Alga	Treatment	Atom % excess ^{15}N in alga	^{15}N enrichment over alga grown in untreated sea water
Calothrix	No addition	0·364	—
		0·342	
	+ phosphate	0·424	+0·062
		0·405	
	+ trace elements	0·414	+0·067
		0·426	
	+ phosphate and trace elements	0·524	+0·169
		0·519	
Nostoc	No addition	0·361	—
		0·365	
	+ phosphate	0·390	+0·046
		0·427	
	+ trace elements	0·383	+0·048
		0·438	
	+ phosphate and trace elements	0·491	+0·134
		0·503	

Period of exposure = 3 hr; labelling of gaseous N = 60 atom % ^{15}N; volume of medium = 25 ml.; volume of gas = 15 ml.; light intensity = 600 foot-candles; mean temperature = 25°; mean ^{15}N content of inocula = 0·372 atom %.

waters, fixation being greatest in January samples and lowest in July samples. Fixation in April and October samples was of the same order. To determine whether fixation in July samples could be improved, additions of 50 mg./l. of K_2HPO_4 and of trace elements similar to those used previously (Stewart, 1962) apart from a further addition of 60 μg./l. of Co (as $CoCl_2$) were made. The results (Table 5) show that fixation was increased on the addition of either supplement, the addition of both simultaneously resulting in greater fixation than either alone.

The effect of combined nitrogen on fixation of elemental nitrogen. The inorganic combined nitrogen concentrations in coastal waters are generally in the region of 10–100 μg./l. (Harvey, 1960), but blue-green algae often predominate where combined nitrogen is relatively high (e.g. areas rich in bird excreta, polluted bays and estuaries). The algae were therefore grown in the presence of 50 mg./l. of ammonium-nitrogen, a concentration unlikely to be exceeded in Nature.

The data obtained (Table 6) show that in the presence of such a concentration nitrogen fixation by Calothrix and Nostoc is inhibited by approximately 86 % and 90 % respectively.

Table 6. *The effect of ammonium-nitrogen on fixation of elemental nitrogen by Calothrix and Nostoc*

Alga	Sample	Total N assimilated by alga (μg.)	Atom % ^{15}N in N assimilated by alga	N in alga from medium (μg.)	N in alga from atmosphere (μg.)	Atmospheric N fixed as percentage of total N assimilated	
Calothrix	1	788	4·590	691	97	12·3	
	2	960	4·351	798	162	16·9	(13·9)
	3	828	4·585	725	103	12·4	
Nostoc	1	1102	4·677	984	118	10·7	
	2	1113	4·865	1034	79	7·1	(9·8)
	3	1189	4·625	1050	139	11·7	

N in Calothrix inoculum = 50 μg.; ^{15}N content of Calothrix inoculum = 0·372 atom %; N in Nostoc inoculum = 132 μg.; N content of Nostoc inoculum = 0·370 atom %; ^{15}N content of NH_4-N supplied = 5·236 atom %; figures in parentheses are means.

DISCUSSION

It is obvious that both Calothrix and Nostoc assimilate elemental nitrogen, thus confirming the long-term growth analyses of Stewart (1962, 1963). The data on fixation in natural sea-water suggest that both species may contribute appreciable quantities of fixed nitrogen to their natural environments. The greater fixation in natural sea-water compared with artificial medium is not a salinity effect, for Fig. 1 shows growth at 0·6 % (w/v) to be similar to that at 3·5 % (w/v). It may be a result of organic material in natural sea-water stimulating growth, although the algae have been grown routinely for the past 5 years in purely mineral medium and, like other nitrogen-fixing Myxophyceae (Fogg, 1956), appear to have no organic growth-factor requirement. A second possibility is that the combined nitrogen in natural sea-water reduces the lag phase which occurs when these species are transferred to nitrogen-free medium.

The data in Fig. 1 indicate that the salinity levels generally occurring in Nature

should not markedly affect growth and fixation. Calothrix indeed appears to be one of the most euryhaline algae isolated in pure culture. Its ability to withstand high salinity without growing is a feature noted for other algae, e.g. *Peridinium balticum* (Provasoli, McLaughlin & Pinter, 1954). Extrapolation of the Nostoc data indicates that this alga should grow in freshwater habitats and its occurrence there has been noted (Bornet & Flahault, 1888). The ability of certain blue-green algae to fix nitrogen both in marine and freshwater environments is probably a more common phenomenon than generally realized. In addition to the above data Allen (1958) reported that the freshwater blue-green algae *Anabaena cylindrica*, *Calothrix parietina* and various Nostoc species fixed nitrogen at salinities approaching those of natural sea-water. Fay & Fogg (1962) reported similarly for *Chlorogloea fritschii*.

The tolerance of Calothrix and Nostoc to large salinity changes accords with their niche on the supra-littoral fringe of the seashore. Ércegovic (1930) recorded growth of *Calothrix scopulorum* in the field at salinities of 0·4–3·8 % (w/v) but not where salinities varied from 0 to 28·3 % (w/v). The present data suggest that lack of growth in the latter habitat may be due more to the high salinity levels encountered rather than to the low salinities. Thus, an increase in salinity may be partly responsible for the disappearance in summer of a large proportion of the British marine blue-green algal flora.

The data on production of ^{15}N-labelled extracellular nitrogen indicate that this is a general phenomenon associated with these species. The lower labelling of the medium shows that all the nitrogen liberated is not specifically associated with the fixation process, this suggestion being supported by the findings (Fogg, 1952; McCalla, 1963) that non-nitrogen-fixing algae also liberate extracellular nitrogen. Zelitch, Rosenblum, Burris & Wilson (1953) found that *Clostridium pasteurianum* liberated extracellular nitrogen more highly enriched with ^{15}N than the intracellular nitrogen after 45 min. exposure to the isotope. The labelling of extracellular nitrogen by the present algae during the early stages of fixation is being investigated.

All the sea-water samples tested supported nitrogen fixation (Table 4); thus throughout the year the marine environment should allow both algae to fix nitrogen. The lower fixation in July samples may be correlated with the poorer supply of inorganic nutrients in the sea during the summer months. Inorganic nutrients limiting marine productivity are phosphorus, nitrogen and trace elements (Harvey, 1960). Thus, with nitrogen-fixing organisms nitrogen should not be limiting providing the other essential nutrients are in adequate supply. The Table 5 data show that in sea-water phosphate and at least some of the trace elements are present in suboptimal concentrations for fixation, a conclusion also arrived at by Allen (1963) from studies on algal nitrogen fixation in artificial sea-water. The concentrations presently employed are not necessarily optimum and probably greater stimulation of fixation by these elements may be achieved.

It appears to be a general phenomenon that combined nitrogen (particularly ammonium-nitrogen) inhibits nitrogen fixation. This suggestion is supported by the data in Table 6. The incomplete inhibition of fixation may be due in part to the high salinity of the medium (3·5 %, w/v), for there is evidence (Stewart, unpublished) that the toxic effect of high ammonium-nitrogen levels on growth of these organisms may be reduced by increasing the salinity of the medium.

These data suggest that in Nature some fixation of nitrogen is likely to be associated with these species.

The author is indebted to Professor G. E. Fogg for his encouragement and helpful advice, to Dr P. Fay for direction in the use of the mass spectrometer and to Miss Gail Hepburn for technical assistance. The mass spectrometer used was purchased with the aid of a grant to the Department from the Department of Scientific and Industrial Research. The award of a grant-in-aid from The Royal Society is also gratefully acknowledged.

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J. gen. Microbiol. (1964), 34, 423–428
Printed in Great Britain

Genetic Analysis of Oxytetracycline Resistance in *Bacillus subtilis* by means of Transformation

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(*Received* 9 *April* 1964)

SUMMARY

Mutants of *Bacillus subtilis* resistant to oxytetracycline were produced and DNA prepared from them was then used for transforming a sensitive strain. DNA from a first-step mutant seemed to transmit a single genetic factor. On the other hand, bacteria, transformed with DNA from a second-step mutant, showed a trimodal distribution of resistance, suggesting that at least two factors were involved in the process. Transformation with DNA from clones belonging to the first and second peaks of the distribution showed that such clones transmitted a single genetic factor for oxytetracycline resistance, while DNA from clones of the third peak transformed sensitive bacteria in a way similar to that of the second step mutant. The results suggested that the two factors are linked and that they had a cumulative effect on drug resistance.

INTRODUCTION

Drug resistance in micro-organisms is known to be under genetic control. Two main types of chromosomal resistance have been described: one-step resistance, when only one mutation seems to be sufficient to permit the bacterium to resist high doses of drug, as in the case for streptomycin (Demerec, 1948); and stepwise or multi-step resistance, when organisms need more than one mutation to increase their resistance, as for penicillin (Demerec, 1945) and chloramphenicolr esistance (Cavalli & Maccacaro, 1950). One gene is presumed to control the mechanism of resistance in the first type, many genes in the second. The genetic analysis of drug resistance has also been approached by means of transformation. Hotchkiss (1951), working with Pneumococcus, obtained three different degrees of penicillin resistance by transformation with DNA from a multi-step highly resistant strain. His results were in agreement with the suggestion by Demerec (1945) that multi-step drug resistance in bacteria originates in successive mutational steps. Later on Hotchkiss & Evans (1957) reported detailed studies on genetic control of sulphonamide resistance in a highly resistant mutant of Pneumococcus. They showed that the high sulphonamide resistance in that strain was under the control of three linked units.

Ravin & Iyer (1961), studying erythromycin resistance in five different spontaneous mutants of Pneumococcus by means of transformation, concluded that three distinct degrees of resistance were present in those mutants; the genetic factors were linked and different combinations of the mutations might have either an antagonistic, synergistic or non-synergistic effect on the phenotypic expression of the resistance.

In a mutant of *Haemophilus influenzae* resistant up to 700 μg. streptomycin/ml.

Hsu & Herriott (1961) found that resistance was controlled by at least two unlinked genes. These two genes, when separated in different bacteria, gave a resistance to 10 and 100 μg. streptomycin/ml., respectively. When they entered the same bacterium, there was an almost multiplicative effect and the organism resisted up to 700 μg. streptomycin/ml. In the present paper are reported some results on the genetic analysis of the stepwise resistance to oxytetracycline (OT) in *Bacillus subtilis*.

METHODS

Media. Difco Bacto-Penassay; Spizizen minimal medium (1958) for preparing competent bacteria; medium 40 (Magni & von Borstel, 1962), a minimal solid medium adjusted to pH 7, always fortified with tryptophan (20 μg./ml.) and histidine (20 μg./ml.) for all platings.

Table 1. *Mutants of Bacillus subtilis used*

The meaning of the symbols is as follows: *try*, requirement for tryptophan; *his*, requirement for histidine; OT^r, resistance to oxytetracycline.

Strain	Genotype	Derivation
SB25	his_2 try_2	Nester & Lederberg (1961)
SB57	his_2 try_2 $OT-r$ (120 μg./ml.)	From strain SB25 by selection on medium 40 + oxytetracycline 40 μg./ml.
SB151	his_2 try_2 $OT-r$ (250 μg./ml.)	From strain SB57 by selection on medium 40 + oxytetracycline 150 μg./ml.
SB151/1	his_2 try_2 $OT-r$ (120 μg./ml.)	From strain SB25 treated with DNA from strain SB151
SB151/5	his_2 try_2 $OT-r$ (160 μg./ml.)	
SB151/8	his_2 try_2 $OT-r$ (250 μg./ml.)	

Selection of oxytetracycline-resistant mutants of Bacillus subtilis. A culture of *Bacillus subtilis* SB25 (kindly supplied by Dr J. Lederberg), grown in Penassay broth at 37° for 15 hr, was centrifuged and resuspended in saline at a concentration of 4×10^9 bacteria/ml.; 0·1 ml. samples of this suspension were plated on medium 40 supplemented with 40 μg. oxytetracycline/ml. (in this medium strain SB25 is inhibited by 15 μg. oxytetracycline/ml.). After incubation for 48 hr at 37°, 50 colonies were picked and streaked on medium 40 supplemented with different concentrations of oxytetracycline. The 50 tested colonies showed, after 24 hr, normal growth on medium containing up to 120 μg. oxytetracycline/ml.; these were presumed to be first-step mutants. Second-step mutants, resistant to about 250 μg. oxytetracycline/ml., were derived from a first-step mutant, SB57, also by selection on solid medium, containing 150 μg. oxytetracyline/ml. All the degrees of resistance were tested by the streak technique.

DNA preparation, transformation procedure and transformant selection. DNA from donor resistant mutants was extracted by following the technique described by Ephrati-Elizur, Srinivasan & Zamenhof (1961). Competent bacteria of the recipient strain were prepared according to the procedure of Young & Spizizen (1961). Competent bacteria were treated with 0·1 μg. DNA/ml.; after incubation for 30 min. deoxyribonuclease (Sigma; 20 μg./ml.) was added, and bacteria were plated after a further 60 min. The selective medium was supplemented with 40 μg. oxytetracycline/ml.,

the minimal inhibitory concentration, in order to recover all the resistant transformants. The degree of resistance of colonies grown on selective medium was individually tested on different concentrations of oxytetracycline.

RESULTS

According to the procedure described in the preceding paragraph, the strain SB25 of *Bacillus subtilis* was transformed with DNA from strain SB57 obtained by a single step of selection (resistant to 120 μg. oxytetracycline/ml.) and with DNA from strain SB151 obtained from the latter by means of a second step of selection and resistant to about 250 μg. oxytetracycline/ml. All the transformed bacteria were selected, in both experiments, on medium 40 containing 40 μg. oxytetracycline/ml. and afterwards many of the transformant colonies were tested for their resistance to this compound (Table 2). The efficiency of transformation of these strains is reported in the first two lines of Table 2 and the distribution of the resistance of the transformant colonies is shown in Figs. 1 and 2, respectively.

From Fig. 1 it can be seen that the transformants with DNA from the strain made resistant by a single step were very uniform, as they showed a single-peaked distribution with the mode at 120 μg. oxytetracycline/ml., repeating exactly the phenotypic distribution of resistance of strain SB57. On the other hand the transformants with DNA from the strain SB151, obtained by two steps of selection, showed (Fig. 2) a trimodal distribution with modes at the first, second and third peaks of oxytetracycline 120, 160 and 250 μg./ml., respectively. From the genetic point of view, these results might be interpreted as due to two factors with a cumulative effect, or to three factors not showing any synergistic properties.

Table 2. *Transformation for oxytetracycline resistance with DNA from different resistant mutants of Bacillus subtilis*

	Efficiency of transformation		Controls without DNA		Distribution of resistance among transformant colonies
Donor strains	No. transformant colonies	No. plated bacteria	No. resistant colonies	No. plated bacteria	
SB57 *OT−r* (120 μg./ml.)	268	$1{\cdot}2\times10^{6}$	1	$1{\cdot}2\times10^{8}$	See Fig. 1
SB151 *OT−r* (250 μg./ml.)	2842	$8{\cdot}8\times10^{7}$	0	$6{\cdot}9\times10^{7}$	See Fig. 2
SB151/1 *OT−r* (120 μg./ml.)	820	$2{\cdot}8\times10^{7}$	0	$5{\cdot}4\times10^{7}$	See Fig. 3
SB151/5 *OT−r* (160 μg./ml.)	1786	$2{\cdot}4\times10^{7}$	0	$1{\cdot}4\times10^{8}$	See Fig. 4
SB151/8 *OT−r* (250 μg./ml.)	612	$1{\cdot}1\times10^{7}$	0	$1{\cdot}4\times10^{8}$	See Fig. 5
SB151/1 + SB151/5	1255	$3{\cdot}9\times10^{7}$	2	4×10^{8}	See Fig. 6

Transformation for resistance to oxytetracyline with DNA from transformant colonies. Three clones obtained in the experiment shown in Fig. 2 were used as donor strains. The clones were: SB151/1 from the first peak (resistant to 120 μg. oxytetracycline/ml.); SB151/5 from the second peak (resistant to 160 μg. oxytetracycline/ml.); SB151/8 from the third peak (resistant to 250 μg. oxytetracycline/ml.).

The efficiency of transformation with DNA extracted from these clones is reported in the third, fourth, fifth and sixth lines of Table 2. The distribution of the resistance between clones is shown in Figs. 3, 4, 5.

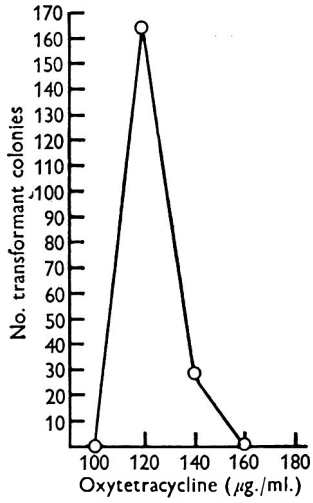

Fig. 1

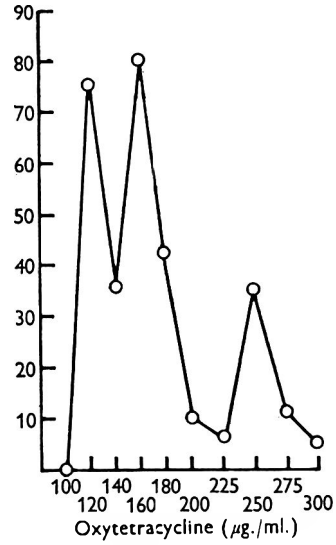

Fig. 2

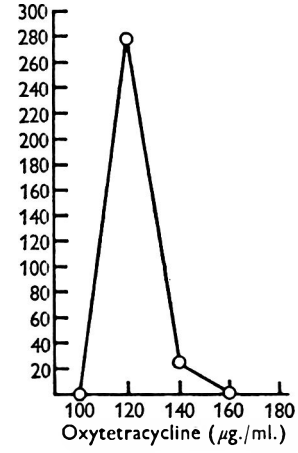

Fig. 3

Fig. 1. Distribution of resistance between 192 transformant colonies, when DNA from the first-step resistant mutant was used.

Fig. 2. Distribution of resistance between 298 transformant colonies, when DNA from the second-step resistant mutant was used.

Fig. 3. Distribution of resistance between 300 transformant colonies when DNA from the first peak (Fig. 2) was used.

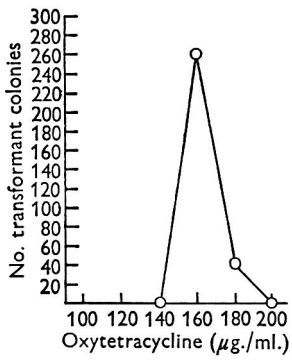

Fig. 4

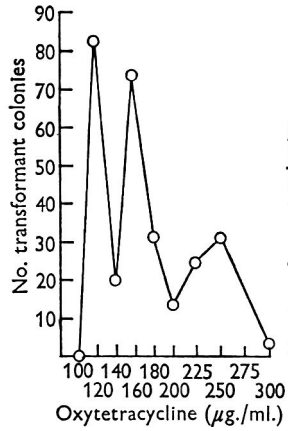

Fig. 5

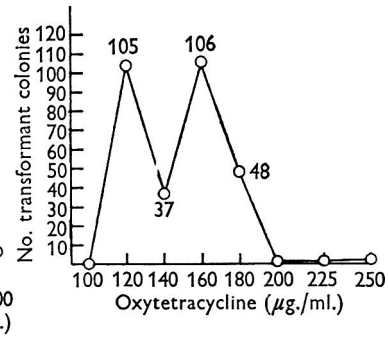

Fig. 6

Fig. 4. Distribution of resistance between 300 transformant colonies, when DNA from the second peak (Fig. 2) was used.

Fig. 5. Distribution of resistance between 300 transformant colonies, when DNA from the third peak (Fig. 2) was used.

Fig. 6. Distribution of resistance between 300 transformant colonies when a mixture of two DNAs (from first and second peaks) was used.

From this, it can be seen that the transformants with DNA from the first peak gave the same distribution as strain SB 57, a single-step resistant mutant, with mode at 120 μg. oxytetracycline/ml. Also the clone of the second peak seemed to produce a genetically uniform DNA, which transformed for a modal resistance of 160 μg. oxytetracycline/ml. The use of a mixture of the DNAs obtained from the first and second peak gave a bimodal resistance distribution (Fig. 6) with modes corresponding to the two original peaks and only four colonies out of 300 outside the distribution. For the present analysis the distribution of the resistance of the transformants with DNA from the third peak is very interesting, since it repeats the same trimodality already shown in Fig. 2, but by using as donor the DNA from the second-step resistant mutant.

DISCUSSION

In the above experiments it seems that two mutations are involved for resistance to oxytetracycline; the first mutation determines an average of resistance to oxytetracycline of about 120 μg./ml. The fact that the first step of resistance might be due to a mutation in a single gene shown from the absence of segregation for resistance when the DNA of strain SB 57 of *Bacillus subtilis* or of the first peak of the trimodal distribution (Fig. 2) is used. In the second selection cycle for resistance to oxytetracycline, a second mutation was selected, which was synergistic to the first. This new mutation when present alone in a strain caused an average resistance to oxytetracycline 160 μg./ml. and when associated with the first mutation added its resistance to it.

These facts are proved by the genetic uniformity of the DNA from the second peak clone (SB 151/5), while, from the two-step resistant strain (SB 151) and from the clone SB 151/8 of the third peak, three modes of resistance appear (Fig. 2). Clear evidence of non-random segregation of the two factors also comes from Figs. 2, 5 and 6. In fact, during the experiment, when a mixture of two separate preparations of DNA were used (each one carrying one of the two factors) only four colonies out of 300 showed a resistance of the order of magnitude which one would have expected from the simultaneous presence of two factors. However, in the two cases in which the DNA of the strain resistant to 250 μg. oxytetracycline/ml. and of the transformant resistant to 250 μg./ml. was used, the colonies which were highly resistant were, respectively, 67 of 298, and 81 of 290. These facts seem to show a 25% co-transfer of the two factors. It can therefore be concluded that the resistance to oxytetracycline, at least within the limits of the two steps studied in this experiment, is due to two mutations which are closely linked and possess almost complete additivity at the phenotypic level.

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J. gen. Microbiol. (1964), **36**, 429–439
Printed in Great Britain

Farmer's Lung Disease: the Development of Antigens in Moulding Hay

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(*Received* 13 *April* 1964)

SUMMARY

Antigens important in farmer's lung disease (FLH antigen complex) developed in wet hay ($\geqslant 30\%$ water content) 4–6 days after baling; their development was associated with increases in pH value of the hay, in content of soluble and volatile nitrogen, and in numbers of actinomycetes, bacteria and fungi. Most of the antigens were common to the actinomycetes *Thermopolyspora polyspora* and *Micromonospora vulgaris*, but others were unidentified. Brown hay from a self-heated stack contained no FLH antigen except where actinomycetes and fungi had developed and the pH had risen from 4·5 to near 7·0.

INTRODUCTION

A collaborative investigation into the nature and mode of origin of 'farmer's lung hay' (FLH) antigen, responsible for farmer's lung disease, has been undertaken at Rothamsted (microbial and biochemical changes in moulding hay) and at the Institute of Diseases of the Chest, Brompton (immunological studies of the antigens in the hay). Two batches of moist baled hay and one moist stack (*A*, *F*, *SA*, respectively) with their corresponding drier controls (*B*, *G*, *SB*) were described by Gregory, Lacey, Festenstein & Skinner (1963); an account of the immunological properties of these hays is now given. Further details of the microbial and biochemical changes in batches *F* and *G* are also presented.

The diverse microflora of mouldy hay (Gregory & Lacey, 1963), is a source of many antigens. In previous investigations Pepys, Riddell, Citron & Clayton (1962) showed that mouldy hay contains fungal and other antigens for which precipitins are present in the sera of patients with farmer's lung disease.

Antigens from thermophilic actinomycetes, especially *Thermopolyspora polyspora* and *Micromonospora vulgaris*, have been found in many mouldy hays, and *T. polyspora* is the most important source so far found of 'farmer's lung hay' (FLH) antigen which appears to be relevant to the production of farmer's lung (Pepys *et al.* 1963*a*). Fungal antigens, mainly a complex of several components common to species of Mucor and *Cladosporium herbarum*, were present in almost all mouldy hays examined but they seem not to be important in farmer's lung. The term 'fungal antigen'

is used in this sense throughout this paper. The antigens derived from these fungi and actinomycetes have provided the basis for the analysis of the antigens which appeared in serial samples of the hays *F*, *A* and *SA* in the course of moulding.

METHODS

Field experiments. A mixed grass ley from Great Knott I Field at Rothamsted was cut on 19 June 1961, and part of the hay baled at 35 % water content on the following day (*F*). A second batch (*G*), the drier control, was baled at 20 % water content on 21 June. The making of the other batches of hay has been described by Gregory *et al.* (1963).

Microbial and biochemical changes were studied by the methods described by Gregory *et al.* (1963) in samples of hay collected almost daily up to 20 days, with a final sample on the 27th day. Part of each sample was de-fatted with acetone within an hour or two of collection, air-dried and stored until needed. A phenol saline extract was then made, dialysed, Seitz filtered and freeze dried. The freeze-dried material was dissolved in 0·9 % sodium chloride for the precipitin tests.

Immunological tests. Hay extracts were tested for precipitin reactions in agar gel against a set of 30 sera, using the double-diffusion method of Ouchterlony, and by immuno-electrophoresis. All the sera gave precipitin reactions to extracts of a mouldy hay H44 (Gregory & Lacey, 1963) in the double-diffusion test. The sera were used in four groups of 16, 5, 5, and 4 sera, respectively, and the groups numbered 1–4 in that order.

The double-diffusion tests were made in Petri dishes with 1·5 % 'Oxoid' Ionagar no. 2 in 0·9 % sodium chloride solution, to which was added 0·1 % sodium azide. In each dish a large central well was used for the test serum and six smaller peripheral wells for the test extracts (10 mg./ml.). The plates were incubated at 28° for 5–7 days.

Immuno-electrophoresis tests were made in films of 1 % agar gel in 'Veronal' buffer (pH 8·75, $\mu = 0{\cdot}025$) on slide coverglasses 8 × 8 cm.; a modification of the micro-method of Scheidegger (1955). The hay extracts were used in a concentration of 30 mg./ml. for electrophoresis for 75 min. with a potential gradient through the gel of approximately 4·5 V/cm. The test sera were then placed in troughs cut into the gel along the line of the electrophoresis, and the slides incubated at 28° in a humid, atmosphere overnight.

Inhibition tests. Inhibition tests were used to identify the antigens responsible for the various precipitation lines. The freeze-dried extracts of mouldy hay H44, and of cultures of fungi and thermophilic actinomycetes, respectively, were added to the appropriate sera to a concentration of 10 mg./ml. After 2 hr at room temperature the mixture was centrifuged to remove the antigen-antibody precipitate, and the absorbed serum tested by double-diffusion to see whether complete inhibition was produced against the other extracts.

RESULTS

Microbial and biochemical changes in hays F and G

Microbial changes. In hay *F*, increases in the numbers of actinomycetes and bacteria paralleled increases in fungi, as determined by the cascade impactor (Gregory & Lacey, 1963). Numbers in both groups exceeded 10 million/g. at 5 days after baling

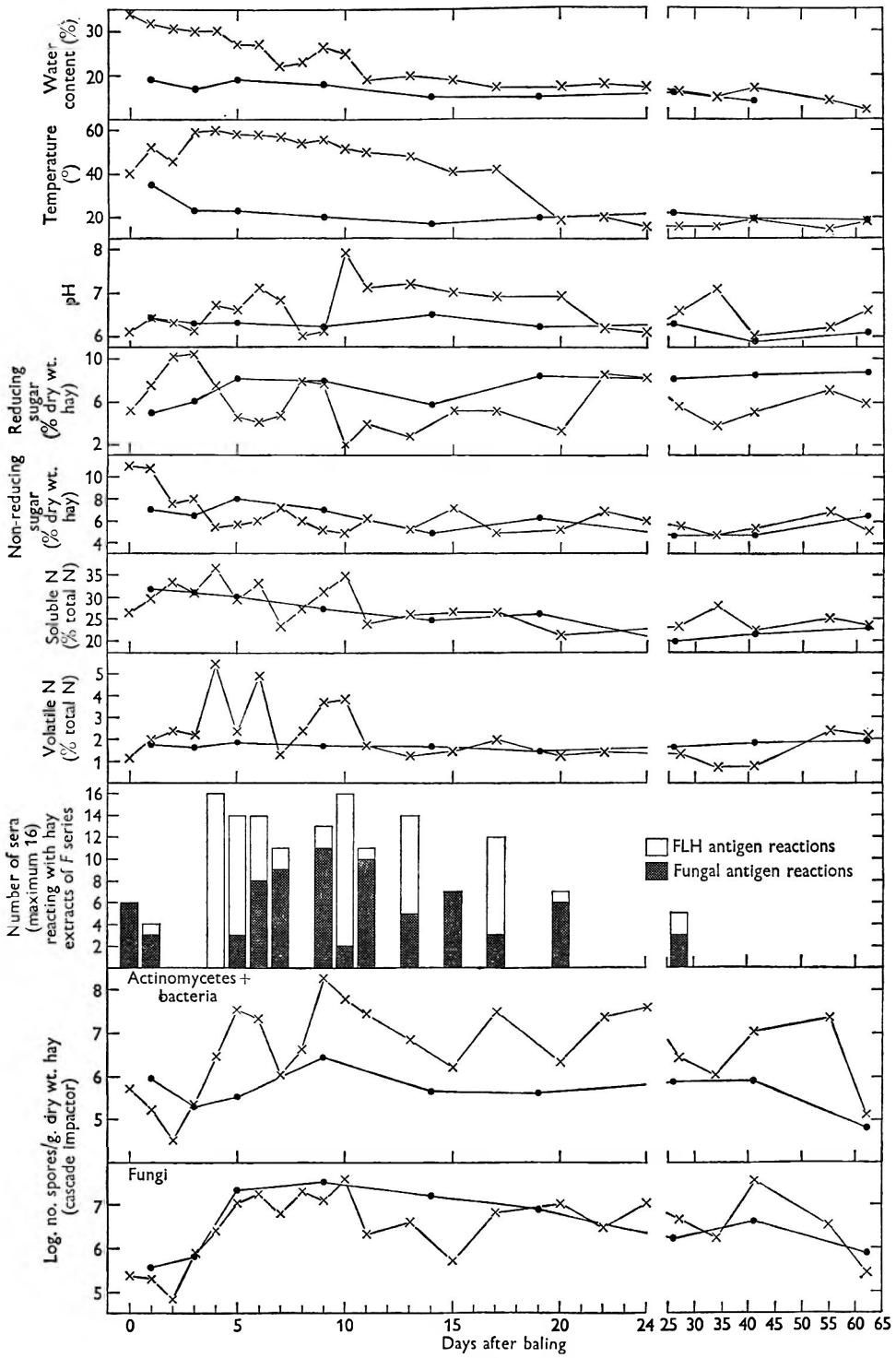

Fig. 1. Changes in wet (*F*) and dry (*G*) baled hays of Great Knott I field, made in June 1961. Development of FLH and fungal antigens in hay of the *F* series is shown by the histogram. ×, wet bales (*F*); ●, dry bales (*G*).

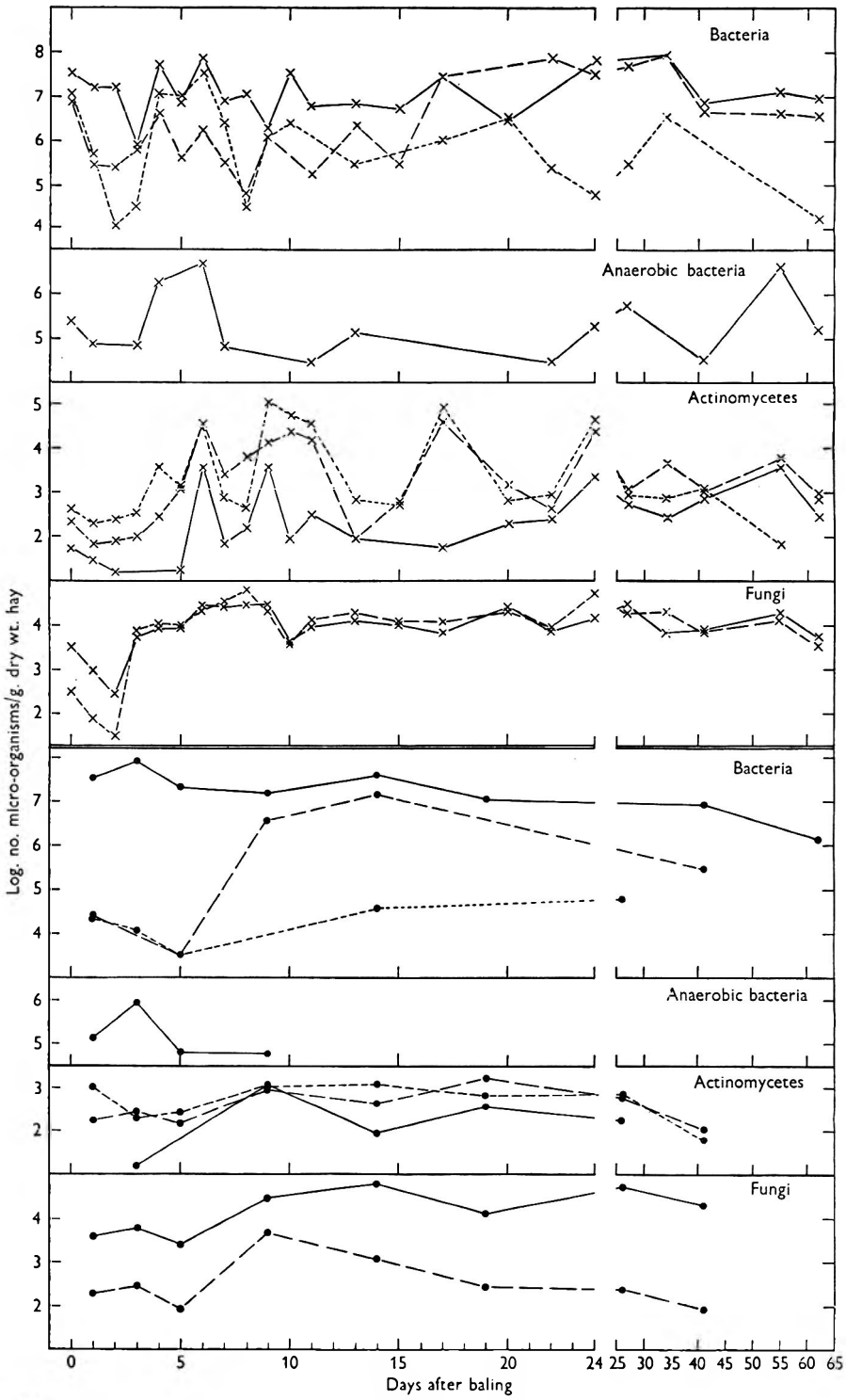

Fig. 2. Changes in the micro-flora of wet (*F*) and dry (*G*) baled hays of Great Knott I field, made in June 1961. ×, Wet bales (*F*); ●, dry bales (*G*). ———, 25°; — — —, 40°; ---------, 60°.

(Fig. 1), that is, 2–3 times as many as in the *A* bales, in which numbers reached a maximum 8 days after baling. Micro-organisms in both groups remained abundant throughout the 8-week sampling period.

Bacteria able to grow at each incubation temperature (25°, 40° and 60°) were numerous throughout the sampling period, especially those growing at 25° (Fig. 2). At the start there were fewer bacteria able to grow at 40°, but their numbers increased to equal those growing at 25° by the 17th day. Bacteria growing at 60° were most numerous after 6 days and declined thereafter as the bales slowly cooled. Numbers of thermophilic fungi growing at 40° paralleled those at 25°; there was a sharp initial fall followed by a rapid rise to a maximum on the 6th day, after which they remained numerous. Numbers of actinomycetes fluctuated widely, a probable reflexion of uneven drying of the hay, but those growing at 40° and 60° followed roughly the same course and were more numerous than the mesophilic types growing at 25°. Anaerobic bacteria at 35° were detected in many samples: they were numerous on the 6th day but declined thereafter.

The *G* bales contained many fungus spores (Fig. 1) chiefly of *Aspergillus glaucus*, a mesophilic mould. This finding was confirmed by plate counts; numerous fungi grew at 25° but only a few at 40° (Fig. 2).

Biochemical changes. The *F* bales (Fig. 1) showed a loss of reducing sugar and rise in pH value. The decrease in pH value after baling, shown by some of the *A* samples, did not occur, probably because of the rapid increase of fungi, which is also reflected in the early decrease in reducing sugar. Non-reducing sugar (fructosan, sucrose) changed little after the initial rapid decrease, in contrast to *A* samples, where it decreased steadily with time. The changes in glucose followed those of total reducing sugar, but the fluctuations were greater, suggesting that glucose was utilized more readily than fructose.

Soluble N, estimated by hot-water extraction (Fig. 1), increased at first and then decreased, as also did soluble N estimated by acid extraction (acid-soluble N); acid-soluble N was 3–7 units less than the values shown in Fig. 1 for the 4th and subsequent *F* samples; samples with pH $\geqslant 6{\cdot}9$ showed the largest differences. Changes in volatile N followed those of soluble N (Fig. 1) and also correlated with numbers of actinomycetes growing at 60°. The changes in the *G* bales were small (Fig. 1); soluble N decreased with time, possibly associated with the development of *Aspergillus glaucus*.

Immunological results for the F series

The extract of each hay sample was tested by double-diffusion against the 16 sera of Group 1. The number of sera giving positive reactions with the samples increased sharply to the maximum of 16 for the 4th day sample. From 11 to 15 sera reacted with the subsequent samples, and there was a decrease in the number of reacting sera after the 17th day. Occasional samples, such as those of the 2nd and 15th days, reacted with few sera, 0 and 7, respectively. Micro-organisms were more numerous in those hays giving many reactions than in those giving few (Figs. 1, 2).

Inhibition tests with fungal and mouldy hay extracts. Sera giving positive precipitin reactions were treated with extracts of a species of Mucor or of *Cladosporium herbarum* to absorb the fungal antibodies and then re-tested against the hay extracts. Complete inhibition of the reaction of a particular serial sample of hay thereby indicated the

presence of fungal antigens only in that sample. Reactions of some samples were not inhibited, or were only partially inhibited, showing that some other antigens were present. These inhibition tests showed that chiefly fungal antigen was present in the hays of days 0 and 1 (Table 1). The 4th day sample gave reactions with all 16 sera but no reactions were completely inhibited by extracts of the fungi. Thus, additional antigens were present in this hay extract though the presence of some fungal antigen as well was not precluded by this test. Complete inhibition of the reactions was,

Table 1. *F series. Precipitation reactions of* 16 *sera with extracts of hay samples*

Days after baling	0	1	2	4	5	6	7	9	10	11	13	15	17	20	27
Total no. of serum reactions (max. 16)	6	5	0	16	14	14	11	13	16	11	14	7	12	7	5
*Complete inhibition. Absorption of serum by Mucor species	6	4	0	0	3	3	9	11	1	10	3	5	2	6	3
Cladosporium herbarum	3	4	0	0	0	2	1	1	1	1	2	5	1	5	2
Reactions not completely inhibited by fungal extracts. Complete inhibition by mouldy hay extract (H 44)	0	1	0	16	11	6	2	2	14	1	9	0	9	1	2
pH value	6·1	6·4	6·3	6·7	6·6	7·1	6·8	6·1	7·9	7·1	7·2	7·0	6·9	6·9	6·6
Bale temperature (°C)	40	52	46	60	58	58	57	56	52	52	49	41	23	20	20

* Sera completely inhibited by Mucor or *C. herbarum* are not necessarily the same.

Table 2. *F series. Inhibition tests with* 5 *sera that react with the extracts of hay samples*

Days after baling	0	1	4	5	6	7
Absorption of serum with						
T. polyspora	0	0	5	3	1	1
Mucor species	4	4	5*	2*	5*	1*

* Partial inhibition, all others complete inhibition.

however, obtained when the sera were absorbed with an extract of mouldy hay H44 before re-testing the serial extracts. These additional antigens include FLH antigens, as shown by tests on the sera of patients with farmer's lung, using extracts of mouldy hays known to have caused disease (Pepys *et al.* 1962), and by the production of farmer's lung reactions in affected subjects who were tested by inhalation of these extracts by Williams (1963). The presence of FLH antigens in the 4th-day sample and subsequent samples was confirmed by the other tests described below. The inhibition tests also showed that, for these farmer's lung sera, the Mucor extract contained more fungal antigen than the extract of *C. herbarum*, and that common antigens are present in both of these fungi as described by Pepys *et al.* (1963*b*).

Inhibition tests with extracts of fungi and of Thermopolyspora polyspora. Five sera (Group 2) which reacted in the double-diffusion test to the samples of hay taken between the time of baling and the 7th day were absorbed with extracts of the fungi

before the re-testing of the extracts. Further absorption of the sera with extracts of *Thermopolyspora polyspora* before final testing of the sample extracts enabled the presence of FLH and fungal antigens to be demonstrated in these samples. Table 2 shows that the 1st- and 2nd-day samples contained Mucor antigens only, as the reactions of the test sera with them were inhibited completely by absorption with Mucor antigen. The 4th-day sample, however, contained both Mucor and *T. polyspora* (FLH) antigen since some of the reactions it produced with the test sera were inhibited by Mucor and others by *T. polyspora* antigen. The same applies though to a lesser extent to the samples of days 5, 6 and 7.

Table 3. *F series. Common actinomycete antigens in double-diffusion tests with 5 sera*

Days after baling	9	10	11	13	15	17	20	27
Reaction of identity of hay extract with								
T. polyspora	2	5	0	5	0	0	0	0
M. vulgaris	3	4	1	4	0	0	0	0

Common antigens in double-diffusion tests. Extracts of samples from the 9th to the 27th days were tested against the 5 sera of Group 3. In each test the hay extract was flanked by *Thermopolyspora polyspora* extract on one side and by *Micromonospora vulgaris* extract on the other, the three extracts being equidistant from the test serum. The fusion of precipitin lines of the hay sample with those of the thermophilic actinomycetes showed the presence of common FLH antigens in the hay sample. The presence of FLH antigens common to both *T. polyspora* and *M. vulgaris* in the hay samples of the 9th, 10th and 13th days, is shown in Table 3. The 11th-day sample had FLH antigen of *M. vulgaris* only. Table 1 shows that the reaction of the 17th-day sample with 9 of the 16 sera was inhibited by absorption of the sera with an extract of mouldy hay H44 and not by the fungal extract. In spite of this suggestion that the hay contained FLH antigen, no FLH antigens of *T. polyspora* or *M. vulgaris* were demonstrated (Table 3). This apparent absence of the known FLH antigens is an example of the complexity and multiplicity of antigens in mouldy hay.

Immuno-electrophoresis tests. Extracts of the serial samples were tested by immuno-electrophoresis to see whether they contained FLH antigens characteristic of *Thermopolyspora polyspora* (Pepys *et al.* 1963*b*).

T. polyspora antigen was detected in the 4th-, 5th-, 6th-, 10th- and 13th-day samples, the amounts in the 6th- and 13th-day samples being relatively small. This is in accord with the findings for FLH antigen in double-diffusion tests (Table 1) where the 6th- and 13th-day samples reacted with 6 and 9 of the sera respectively, whereas the other samples reacted with 11 to 16 of the sera.

Statistical results. The statistically significant results for the *F* series show that the number of sera giving FLH reactions with sample extracts is positively correlated with volatile nitrogen ($P \leqslant 0{\cdot}02$), number of fungi and soluble nitrogen ($P \leqslant 0{\cdot}05$), and with pH value, and with numbers of actinomycetes and bacteria ($P \leqslant 0{\cdot}1$).

Immunological results for the G series

In contrast to the *F* series, an extract of only one of the serial samples of the control hay *G* reacted with more than 3 of the 16 Group 1 sera, i.e. the 17th-day sample reacted with 6 sera (Table 4). Absorption of the sera that reacted with these extracts showed that all the reactions except those of one of the sera were of fungal origin (see Results for *B* series).

Table 4. *G series. Precipitation reactions of* 16 *sera* (*Group* 1) *with extracts of hay samples*

Days after baling	2	4	6	10	17	24	35
Total no. of serum reactions (max. 16)	0	0	0	2	6	3	3
*Complete inhibition. Absorption of serum by							
Mucor species	.	.	.	2	3	1	1
Cladosporium herbarum	.	.	.	2	4	1	1
Reactions not completely inhibited by fungal extracts. Complete inhibition by mouldy hay extract (H 44)	.	.	.	0	1	1	1
pH value	6·4	6·3	6·3	6·2	6·5	6·3	6·1
Bale temperature (°C)	35	22	22	30	17	22	—

* See Table 1.

Table 5. *A series. Precipitation reactions of* 16 *sera* (*Group* 1) *with extracts of hay samples*

Days after baling	0	2	3	4	5	6	7	8	9	10	11	13	15	19	25	29
Total no. of serum reactions (max. 16)	8	2	9	15	13	15	10	11	16	12	8	12	10	9	9	7
*Complete inhibition. Absorption of serum by																
Mucor species	6	2	8	12	10	3	3	9	1	6	5	6	6	7	7	6
Cladosporium herbarum	5	2	7	5	7	3	2	4	4	4	2	2	3	4	2	3
Reactions not completely inhibited by fungal extracts. Complete inhibition by mouldy hay (H 44)	1	0	0	3	2	11	7	1	12	6	3	6	3	2	1	1
pH value	6·2	6·0	6·0	6·0	5·9	5·9	5·9	6·0	5·5	5·7	6·8	5·8	5·9	6·1	6·1	6·7
Bale temperature (°C)	25	42	41	47	51	51	52	50	48	46	45	45	40	23	22	21

* See Table 1.

Immunological results for the A series

The *A* bales were less mouldy than the *F* bales, and the correlation of FLH serum reactions with the biochemical and microbial findings was significant only at the 10 % level. The strongest positive correlation was with temperature. As in the *F*

series, fungal antigens were present from the start but FLH antigen appeared a little later. FLH antigen was present in the hay of the 6th day, reacting with 11 of the 16 sera (Table 5). The moulding of this hay, as with the *F* hay, did not proceed uniformly throughout the bale, as shown by the fluctuations in the numbers of reactions produced with the serial specimens which in turn corresponded in general with high or low figures for the fungal and actinomycete flora.

Immunological results for the B series

Only the first two samples of the control series *B* reacted with more than one of the 16 sera, the first with 4 and the second with 7 sera (Table 6). Absorption of the reacting sera showed that a single serum throughout was responsible for those reactions which could only be inhibited by mouldy hay, except for the three reactions to the extract of the second sample, where two additional sera were involved. The single serum was responsible for the similar reactions in the *G* series.

Table 6. *B series. Precipitation reactions of* 16 *sera* (*Group* 1) *with extracts of hay samples*

Days after baling	2	3	5	9	14	22	55
Total no. of serum reactions (max. 16)	4	7	0	1	1	1	1
*Complete inhibition. Absorption of serum by							
Mucor species	2	2	.	0	0	0	0
Cladosporium herbarum	2	4	.	0	0	0	0
Reactions not completely inhibited by fungal extracts. Complete inhibition by mouldy hay extract (H 44)	1	3	.	1	1	1	1
pH value	6·1	6·1	6·0	6·0	6·0	6·0	5·8
Bale temperature (°C)	25	24	17	17	15	17	19

* See Table 1.

Table 7. *SA series. Precipitation reactions of* 20 *sera with extracts of hay samples*

* Sample no.	7–10	14	18	25/1 and 2	25/3	25/4
Days after stacking	7–10	16	28	186	186	186
No. serum reactions to hay extracts	0	17	3	4	15	4
pH value	5·3–5·4	6·3	4·5	4·8	7·8	7·4

* See Gregory *et al.* (1963) for further details of samples 25/1–25/4.

Immunological results for the SA series

In this series samples 14 (17 days) and 25/3 (186 days) gave positive reactions in double-diffusion tests with 17 and 15 sera respectively, out of a group of 20 test sera, Group 1 + Group 4, (Table 7). FLH antigen was demonstrated in both specimens by immuno-electrophoresis. These specimens of hay had pH values of 6·3 and 7·8, respectively. By contrast samples 18 (28 days) and 25/1 (186 days) had pH values of

4·5 and 4·8, and gave only 3 and 4 reactions, respectively, thus emphasizing the role of the high pH value which favoured the growth of the actinomycetes (Gregory *et al.* 1963). In general, *SA* samples with low pH values (brown hay from the stack interior) gave few reactions with sera, but where mould had developed and the pH had risen, samples reacted strongly. However, one sample (25/4) which contained many actinomycetes able to grow at 60°, and which had a pH of 7·4, reacted with only 4 sera.

DISCUSSION

When the *F* series hay was baled it was clear that the rapid drying of the grass in the field in hot sunshine had given an uneven product. Neither the individual bales were uniform in their water content, nor were different parts of the same bale. This may have obscured, to some extent, the pattern of microbial and biochemical change with time of storage. The general course of events was similar to that described previously for the *A* bales (Gregory *et al.* 1963) which had also dried unevenly, but because of rain. Figures 1 and 2 show the fluctuations in the trend of the results for these samples; note particularly the water content of the initial samples.

A salient feature of the results is that FLH antigen appears soon after baling; in the *F* series antigen was present in quantity 4 days after baling, and in the *A* series 6 days after baling. The apparent absence of FLH antigen in some of the later samples may be because they were too dry when baled for antigen to develop.

Hay that has heated to a high temperature does not necessarily contain FLH antigen. The stack *SA* reached 65° and some samples of brown hay obtained from its inner core (e.g. 25/1 and 25/2 at 186 days) contained no FLH antigen (Table 7). These samples contained many spore-forming bacteria, but few fungi and actinomycetes (Gregory *et al.* 1963). The antigen developed in this brown hay only where actinomycetes and fungi were present and the pH had risen from 4·5 to near 7·0.

Gregory & Lacey (1962), in an extensive survey of different types of hay, found that most of the hays associated with clinical cases of farmer's lung that they examined had pH values of 6·6 or more. It was found subsequently, in immunological studies on *W* hay of 1960 (Gregory *et al.* 1963), that samples with a high pH value and containing many actinomycetes gave a reaction with farmer's lung serum. The results for the *F* series, which are statistically significant, show that FLH antigen is positively correlated with pH value, soluble and volatile nitrogen and also with numbers of all three groups of organisms studied, actinomycetes, bacteria and fungi. Elucidation of the effects of pH value and groups of organisms by Pepys *et al.* (1963*a*) showed that thermophilic actinomycetes, *Thermopolyspora polyspora* in particular but also *Micromonospora vulgaris*, produce FLH antigen. Fungi produce only fungal antigens; the high pH value produced by fungi favours actinomycete growth, but does not itself produce FLH antigen.

The numbers of thermophilic actinomycetes occurring in hay depends on the water content on baling; *F* hay baled at 30 % water content averaged about 10 million spores/g. and *A* hay, baled at 35 % water content, averaged about 5 million spores/g. The *A* bales gave only a few samples rich in FLH antigen, suggesting that hay baled at less than 30 % water content will not develop FLH antigen readily.

In the two sets of bales, *A* and *F*, volatile N increased and this was associated with increasing numbers of actinomycetes or bacteria; in the *A* series volatile N was

positively correlated with the numbers of bacteria growing at 60° and in the *F* series with the numbers of actinomycetes growing at 60°. FLH antigen was positively correlated with volatile N and also with soluble N for the *F* series. The increase in soluble N may be caused by proteolysis leading to amino acid formation which in turn gives ammonia on deamination.

The total number of fungi reached a maximum later than the actinomycetes and bacteria, and increases in volatile N did not correspond with increases in total numbers of fungi, except for the *A* bales. However, the detailed study of the microbial succession in the *W* bales (Gregory *et al.* 1963) showed that the phycomycetes *Mucor pusillus* and *Absidia ramosa* appeared as early as 2–4 days after baling and reached their maximum numbers on the 11th to 12th day. The first actinomycetes detected in *W* bales were *Thermopolyspora polyspora* and *Micromonospora vulgaris*, on the 6th day (Gregory *et al.* 1963). It may be that the phycomycetes produce localized areas of comparatively high pH value which together with the high temperature favour early growth of thermophilic actinomycetes. The fungi are chiefly responsible for the decline in soluble sugars, particularly the reducing sugars glucose and fructose, and the decline is associated with an increase in pH value; this increase is probably caused by utilization of fatty acids by fungi, as well as by production of volatile N. The decline in soluble N associated with increasing numbers of total fungi in the F bales may be the result of protein synthesis by the fungi. The detection of FLH antigen in *F* and *A* bales 4–6 days after baling is to be expected from the rapid increase in numbers of thermophilic actinomycetes which accompanies the temperature rise after baling.

We thank Mr J. H. A. Dunwoody for statistical analysis, Mr J. R. Moffatt and the Rothamsted Farm staff for making experimental batches of hay, and Miss Frances Ellingham, Miss Margaret Goodson, Miss Marion Sanders and Mr K. E. Fletcher for technical assistance.

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J. gen. Microbiol. (1964), 36, 441–450
With 2 plates
Printed in Great Britain

Composition of the Mucopolymer in Cell Walls of the Unstable and Stable L-Form of *Proteus mirabilis*

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(*Received* 28 *April* 1964)

SUMMARY

Continuous, cell-shaped mucopolymer layers were isolated from cell walls of some unstable and stable L-forms of *Proteus mirabilis*. The chemical compositions of these non-rigid mucopolymers and of normal rigid mucopolymer basal-structures of rod-shaped Proteus bacteria were very similar. Electron microscopy showed that the protein and mucopolymer of the complex 'rigid layers' were organized differently in cell walls of rods and L-form cells. The results suggest that the rigid-layer protein plays a role in the morphogenesis of the mucopolymer.

INTRODUCTION

Great progress has recently been made in the elucidation of the structure of bacterial cell walls. It has been recognized that the rigid layer (R-layer) of the cell wall with its main component, the mucopolymer, plays an essential role in the maintenance of cell shape and rigidity (see Salton, 1960; Martin, 1963*a*; Weidel & Pelzer, 1964). This knowledge provides a chemical basis for understanding the processes which are at work during the conversion of rod-shaped bacteria into fragile globular spheroplasts and L-forms. Studies on the formation of penicillin-induced spheroplasts in some Gram-negative bacteria have shown that inhibition of mucopolymer biosynthesis and autolytic breakdown of the existing R-layers take place here (Salton & Shafa, 1958; McQuillen, 1958; Leutgeb, Pelzer & Schwarz, 1963). However, this interpretation does not seem to be applicable to some of the L-forms of *Proteus mirabilis*. Fragile walls of the unstable (reversible) L-form of Proteus, grown in penicillin medium, were shown to contain normal amounts of mucopolymer with the typical set of amino acids and amino sugars (Kandler, Hund & Zehender, 1958; Martin, 1963*b*). Among the stable (non-reverting) Proteus L-forms Weibull (1958) and Morrison & Weibull (1962) observed at least one strain, L9, which contained a considerable amount of the mucopolymer constituent diaminopimelic acid in the macromolecular cell fraction. It appears then that the mechanical defects of the L-form walls might not be caused by the inability to synthesize mucopolymer and to retain it in the cell walls, but rather by changes in the quantitative composition or in the macromolecular organization of the mucopolymer. The present paper describes the isolation of mucopolymer layers from cell walls of several L-forms and rod-shaped bacteria of *P. mirabilis* and their characterization by chemical analysis and electron microscopy.

METHODS

Organisms. Strain VI of *Proteus mirabilis* and the stable Proteus L-strains LVI and LD52 were obtained from O. Kandler and had originally been received from U. Taubeneck. The unstable L-strain 1 Ca/pen had been isolated in 1955 by Kandler & Kandler (1956). It has been propagated since then by serial transfer on agar medium containing 200 international units (i.u.) penicillin/ml. Other strains of rod-shaped Proteus bacteria and of the stable L-form were derived from the unstable strain 1 Ca/pen in the course of the present work. Their isolation is described below in Results.

Growth conditions. Mass cultures of Proteus bacteria and L-forms were grown at 35° in 8 l. batches of vigorously aerated medium containing the following nutrients per litre: tryptic casein peptone (Merck, Darmstadt), 15 g.; beef extract (Brunnengräber, Lübeck), 15 g.; glucose, 2 g.; NaCl, 5 g.; Na_2HPO_4, 4·5 g.; distilled water; adjusted to pH 7·0. Penicillin (200 i.u./ml.) was added to cultures of 1 Ca/pen. The stable L-strains LVI and LD52 required the addition of 5 % (v/v) defibrinated horse serum for good growth. Solid media for stock cultures and plating were prepared by adding agar (1·2 %, w/v) to the medium. In mass cultures all organisms were grown until the beginning of the stationary phase, the organisms were then harvested in a continuous flow centrifuge, rapidly cooled and stored at −20°.

Isolation of cell walls. The conventional method of shaking organisms with glass beads in a Mickle type disintegrator and subsequent differential centrifugation (Salton & Horne, 1951) was adequate for the isolation of cell walls from rod-shaped Proteus bacteria. The frozen cell paste was thawed and then vigorously stirred with DNAse (Worthington Biochemical Corp.) 0·1 μg./ml. for 10 min. at room temperature. The organisms were then treated in the Mickle apparatus suspended in a 0·8 % (w/v) solution of sodium dodecylsulphate in distilled water to preclude autolytic breakdown of the mucopolymer (Weidel, Frank & Martin, 1960; Weidel, Frank & Leutgeb, 1963). L-form organisms required no mechanical treatment for the separation of cell walls from the cytoplasmic constituents: after thawing and treatment with DNAse those cells were suspended in 0·8 % (w/v) sodium dodecylsulphate solution which caused rapid 'lysis', recognizable by complete clearing of the suspension within 30–60 sec. From the lysates empty or near-empty cell walls were sedimented by centrifugation for 35 min. at 30,000 *g*. Wall sediments were purified by six cycles of washings in distilled water and sedimentation in the centrifuge.

Isolation of mucopolymer layers. The cell-wall layers containing the mucopolymer were isolated from cell walls by means of the phenol extraction procedure of Weidel *et al.* (1960). Following this technique mucopolymer-containing rigid-layers (R-layers) were easily separated from other cell-wall macromolecules in rod-shaped bacteria and L-forms grown in the absence of penicillin. In contrast, mucopolymer layers of the unstable L-form 1 Ca/pen were very light and difficult to separate from the lipopolysaccharide. Repeated cycles of differential centrifugation at 60,000 *g* for 40 min. were required to achieve this separation.

Mucopolymer layers were freed from covalently attached protein by treatment with proteolytic enzymes (Martin & Frank, 1962). Substrates (5 mg. dry weight/ml.) were incubated for 15 hr at 37° with either pepsin (Worthington Biochem. Corp.)

500 μg./ml. in 1/15 M-glycine+HCl buffer (pH 2·5) or trypsin (Worthington Biochem. Corp.) in 1/15 M-Na_2HPO_4 solution.

Analyses of amino acids and amino sugars. Quantitative determinations of amino acids and amino sugars in hydrolysates of mucopolymer layers were made by ion-exchange chromatography with an automatic amino acid analyser (Messrs Bender and Hobein, Munich) as described previously (Martin & Frank, 1962). In preparations which contained large amounts of protein in addition to mucopolymer, methionine was oxidized by treatment with hydrogen peroxide (Dent, 1948) to prevent its interference with the peak of diaminopimelic acid. In these cases parallel runs with hydrogen-peroxide-treated and untreated samples had to be made for the determination of DAP and muramic acid, respectively, since hydrogen peroxide also causes the appearance of an unidentified artifact peak in the position of the muramic acid peak (after serine and before glutamic acid).

RESULTS

Isolation of stable L-forms and rod-shaped revertants from the unstable L-strain 1 Ca/pen of Proteus mirabilis

At the beginning of this work the unstable L-strain 1 Ca/pen of *Proteus mirabilis* had been carried through about 300 weekly transfers on agar medium containing 200 i.u. penicillin/ml. In liquid aerated penicillin medium strain 1 Ca/pen formed spherical forms of different size, average diameter from 1 to 4 μ. Young forms of still smaller size were seen to originate as buds from bigger forms. About 20 % of the spheres were motile. Electron microscopic pictures (Pl. 1, fig. 1) showed forms whose surfaces were densely covered with loosely attached fimbriae and 'spheroidal surface matter' (Brinton, 1959). Some of the forms carried several flagella in a fashion resembling in part the peritrichous flagellation of normal Proteus bacteria.

When strain 1 Ca/pen organisms were plated on penicillin-free nutrient agar very few of them reverted to normal rod-shaped bacteria. Instead, an assortment of colonies with considerable variety in cellular morphology was obtained. The great majority consisted of motile or non-motile organisms of still spherical shape but with sharply defined boundaries (Pl. 1, fig. 2). Pure cultures derived from isolated colonies of such 'stable L-forms' have been subcultured on penicillin-free medium for two years at monthly intervals and have shown no sign of reversion to the bacillary state during this period. These newly isolated stable L-forms closely resembled the original 1 Ca/pen organism in cellular morphology as well as in fragility and sensitivity to osmotic shock.

A few colonies on the reversion plates consisted of oblong organisms of irregular size and shape, others of non-septate filaments of variable length or of regular non-motile rods. Normal motile Proteus bacteria were never obtained from such platings. A detailed account of these observations will be given elsewhere. For further work on cell walls three strains derived from 1 Ca/pen were selected: two stable L-strains (1 Ca/20, 1 Ca/25) and one revertant strain (1 Ca/5) with rod-shaped cells.

Isolation of cell walls from L-forms of Proteus mirabilis treated with sodium dodecylsulphate

Previous observations have shown that the sensitivity of the cell walls of Gram-negative bacteria to disaggregation by an anionic detergent, such as sodium dodecylsulphate, depends on the state of the mucopolymer layer in the walls (Martin, unpublished observations; Weidel *et al.* 1963). The shape and rigidity of cell walls containing intact mucopolymer are not impaired by treatment with sodium dodecylsulphate. In contrast, walls whose mucopolymer has been degraded by autolysins or by lysozyme are rapidly disintegrated by this detergent. Thus, treatment with sodium dodecylsulphate provides a simple method for showing the presence of a continuous mucopolymer layer in a cell wall. When organisms of several L-strains were suspended in solutions of 0·4 % (w/v) sodium dodecylsulphate in distilled water different reactions were observed. Rapid clearing of the suspensions within a few seconds took place in all cases. In 'lysates' of the unstable L-strain 1 Ca/pen and of the stable L-strains 1 Ca/20 and 1 Ca/25 large quantities of empty cell walls were preserved in their original globular shape and size (Pl. 1, figs. 3, 4). Organisms of other stable strains (LVI, LD52) were dispersed completely into minute fragments. Entire empty cell walls were never recovered from the two latter organisms.

The rigid layers in cell walls of rod-shaped Proteus and the rigid layer equivalents of L-form cell walls

Weidel *et al.* (1960) used a phenol extraction procedure to separate the three principal macromolecular components of the cell wall of *Escherichia coli* B: lipoprotein, lipopolysaccharide, rigid layer. When this technique was applied to isolated cell walls of the rod-shaped Proteus strain 1 Ca/5 rigid layers were obtained whose appearance under the electron microscope was very similar to that of the R-layers described earlier for *E. coli* B (Pl. 2, fig. 5). The surface of the Proteus R-layers was densely covered in the same characteristic manner with granular protein matter. This R-layer protein which, unlike the lipoprotein of the cell wall, was not removed by phenol, was solubilized only by treating the R-layers with trypsin or pepsin. Smooth-surfaced cell-shaped mucopolymer 'basal structures' were then obtained (Pl. 2, fig. 6).

When the phenol extraction procedure was applied to isolated cell walls of the unstable L-form 1 Ca/pen, protein + mucopolymer layers were recovered from which treatment with protease liberated heavily damaged mucopolymer membranes (Pl. 2, fig. 7). Yet in spite of their eroded appearance these membranes possessed enough mechanical stability to survive as individual particles, which roughly preserved the contours of the original L-forms. Likewise, bag-shaped rigid-layer equivalents were isolated by extracting cell walls of the stable L-forms 1 Ca/20 and 1 Ca/25 with phenol. These had a more solid texture than the structures present in the unstable L-forms. Before treatment with protease they also contained covalently associated protein and mucopolymer as main components. However, the arrangement of the protein which is typical for R-layers of rods, namely as a granular stratum covering the surface of the mucopolymer basal structure, was never observed here. Complex protein + mucopolymer layers and naked mucopolymer basal

structures, from which the protein had been removed by protease, look very much alike under the electron microscope (Pl. 2, figs. 8, 9).

The isolated mucopolymers from rod-shaped Proteus and from the unstable and stable L-forms were all sensitive to lysozyme. As little as 10 μg. lysozyme/ml. in 1/20 M-ammonium acetate dissolved about 1 mg. dry weight/ml. of all different mucopolymers within a few minutes, leaving no structured residues recognizable in the electron microscope.

Table 1. *Amino acids and amino sugars in 'rigid layers' of rod-shaped bacteria and L-form cells of Proteus mirabilis*

	Bacterial form				L-form					
					Unstable		Stable			
	Proteus mirabilis VI		Strain 1 Ca/5		Strain 1 Ca/pen		Strain 1 Ca/20		Strain 1 Ca/25	
	%	Molar ratio	%	Molar ratio	%	Molar ratio	%	Molar ratio	%	Molar ratio
Diaminopimelic acid	6·8	1·00	7·8	1·00	10·7	1·00	3·1	1·00	6·4	1·00
Glutamic acid	10·5	2·04	8·9	1·52	9·8	1·22	5·8	2·52	8·9	1·85
Alanine	8·1	2·86	7·1	2·20	8·3	1·88	3·9	3·06	6·6	2·49
Muramic acid	4·8	0·51	8·1	0·77	10·4	0·72	2·5	0·59	4·5	0·52
Glucosamine	2·7	0·42	4·6	0·64	9·3	0·94	3·0	1·04	3·2	0·54
Aspartic acid	6·0	1·30	3·9	0·75	4·2	0·59	4·3	2·11	5·9	1·37
Threonine	2·6	0·64	1·6	0·35	1·6	0·26	1·5	0·83	2·3	0·61
Serine	2·5	0·72	2·2	0·57	1·4	0·26	2·1	1·35	2·6	0·78
Glycine	2·2	0·95	0·9	0·36	1·3	0·38	1·4	1·41	1·6	0·74
Valine	3·0	0·75	1·3	0·29	1·2	0·19	2·2	1·25	2·2	0·60
Isoleucine	2·4	0·54	0·8	0·16	0·7	0·10	1·3	0·65	1·8	0·43
Leucine	4·3	0·96	2·1	0·40	1·7	0·25	3·7	1·83	3·7	0·88
Tyrosine	2·9	0·50	1·0	0·13	1·1	0·11	1·5	0·43	1·6	0·26
Phenylalanine	1·5	0·25	1·0	0·15	2·0	0·22	1·5	0·56	2·4	0·43
Lysine	4·7	0·85	1·4	0·24	3·8	0·46	3·0	1·30	3·4	0·71
Arginine	3·0	0·49	1·8	0·25	3·7	0·38	2·4	0·85	2·5	0·43
Total % of dry weight	68·0	—	54·5	—	71·2	—	43·2	—	59·6	—

Chemical composition of 'rigid layers' and mucopolymer basal structures from rods and L-forms of Proteus mirabilis

Quantitative determinations of amino acids and amino sugars were made on samples of rigid layers from two strains of rod-shaped bacteria (*Proteus mirabilis* VI and 1 Ca/5) and on rigid-layer equivalents of unstable and stable L-forms (1 Ca/pen, 1 Ca/20, 1 Ca/25). The results are summarized in Table 1. In all preparations from rods and L-forms typical mucopolymer constituents and a number of 'unspecific' amino acids were found. An interesting difference was observed when liberation of the mucopolymers from the R-layers by means of proteolytic enzymes was attempted. Pepsin was more effective in solubilizing the R-layer protein than trypsin. However, in spite of prolonged enzyme treatment small amounts of amino acids of the order of a few percent adhere tenaciously to all those mucopolymer preparations from rods or from stable L-forms which were formed in the absence of penicillin. In contrast, mucopolymer of the unstable L-form 1 Ca/pen was completely freed from unspecific amino acids by trypsin (Table 2).

Obviously, none of the different basal structure preparations was free from contaminating cell material. Mucopolymer yields were lower in the preparations from the stable L-forms where no effort could be made to apply mechanical methods for obtaining purer cell walls as a starting material. For the same reason no exact figures on yields of cell walls from L-forms can be given now beyond the general statement that, according to microscopic observation, one cell wall and one mucopolymer membrane are obtained from each L-form organism.

Table 2. *Amino acids and amino sugars of mucopolymer basal-structures from bacterial and L-forms of Proteus mirabilis*

Mucopolymer from unstable L-form 1 Ca/pen after treatment with trypsin; all others after treatment with pepsin.

	Bacterial form				L-form					
					Unstable		Stable			
	Proteus mirabilis VI		Strain 1 Ca/5		Strain 1 Ca/pen		Strain 1 Ca/20		Strain 1 Ca/25	
	%	Molar ratio	%	Molar ratio	%	Molar ratio	%	Molar ratio	%	Molar ratio
Diaminopimelic acid	13·4	1·00	11·7	1·00	12·9	1·00	8·3	1·00	11·0	1·00
Glutamic acid	11·0	1·10	10·2	1·16	10·0	1·04	7·3	1·10	8·1	0·99
Alanine	10·6	1·91	9·0	1·87	9·1	1·71	6·3	1·73	6·7	1·50
Muramic acid	15·8	0·87	15·2	0·96	16·7	0·96	1·3	1·00	11·5	0·77
Glucosamine	11·8	0·95	11·3	1·04	12·1	1·01	9·5	1·16	9·4	0·92
Aspartic acid	0·6	0·06	1·2	0·15	—	—	1·0	0·18	1·0	0·13
Threonine	0·3	0·04	0·5	0·07	—	—	0·5	0·09	0·5	0·06
Serine	0·6	0·08	0·7	0·12	—	—	1·3	0·23	0·9	0·16
Glycine	0·4	0·08	0·6	0·15	—	—	0·7	0·23	0·5	0·15
Valine	—	—	—	—	—	—	—	—	—	—
Isoleucine	0·2	0·02	0·4	0·05	—	—	0·4	0·07	0·3	0·04
Leucine	0·3	0·03	0·6	0·08	—	—	0·8	0·14	0·7	0·09
Tyrosine	1·2	0·10	1·2	0·11	—	—	1·0	0·12	1·4	0·13
Phenylalanine	—	—	—	—	—	—	—	—	—	—
Lysine	—	—	—	—	—	—	—	—	—	—
Arginine	—	—	—	—	—	—	—	—	—	—
Total % of dry weight	66·2	—	62·6	—	60·8	—	49·2	—	52·0	—

Absence of mucopolymer from the stable L-form strains LVI *and* LD52 *of Proteus mirabilis*

The disappearance of any insoluble structures resembling cell walls from sodium dodecylsulphate-lysates of the stable L-strains LVI and LD52 strongly suggested that the integuments of this kind of L-form of *Proteus mirabilis* are altogether differently organized. It seems most likely that no covalently interlinked basal structures are present here. It remained to investigate whether organisms of this L-form have incomplete cell walls containing only separate islets of mucopolymer; they might also be completely devoid of mucopolymer or other cell-wall material. The search for mucopolymer was done on hydrolysates of entire organisms of strains LVI and LD52 and also on the small-particle fractions which remained insoluble after lysis with sodium dodecylsulphate, and which were suspected of harbouring mucopolymer fragments. Wet cell sediments of strains LVI and LD52 were frozen and thawed, the paste stirred vigorously for 10 min. with DNAse and then lysed in a 0·4 % (w/v) solution

of sodium dodecylsulphate in distilled water; insoluble material was sedimented from the lysates by centrifugation for 1 hr at 100,000 *g*. The jelly-like sediments were extracted with phenol and the insoluble fractions washed free from phenol with water and freeze-dried. Samples were analysed for amino acids, amino sugars and neutral sugars. Yields of phenol-insoluble matter were 9 % dry weight from strain LD52 and 19 % from strain LVI. The substance from LD52 contained small amounts of unspecific amino acids but not mucopolymer components such as diaminopimelic acid or muramic acid, nor neutral sugars. This material was not further characterized. Mucopolymer constituents were not present in the phenol-insoluble fraction of strain LVI. Components identified by solvent extraction and paper chromatography of hydrolysates were: lipid, galactose, glucose, glucosamine. From this evidence and from the typical appearance of the substance in the electron microscope (see Weidel *et al.* 1960) it is tentatively classified as a lipopolysaccharide. Lipopolysaccharides containing the same sugars have been found in the cell walls of all other investigated *Proteus mirabilis* strains of either rod or L-form, during the isolation of rigid layers by the phenol procedure.

The search for mucopolymer components in hydrolysates of total cell material of strains LD52 and LVI gave entirely negative results. Several sets of samples (20 mg. dry weight) were run on the automatic amino acid analyser. Diaminopimelic acid and muramic acid were looked for in separate samples, with and without oxidation of methionine before application to the ion exchange column (see Methods). No peaks corresponding to diaminopimelic acid or muramic acid were found in any analysis. Since as little as 1/20 μmole diaminopimelic acid can be readily detected by our method, organisms of strains LD52 and LVI must contain less than 0·04 % (w/w) diaminopimelic acid, if any. In contrast, the diaminopimelic acid content determined for bacillary *Proteus mirabilis* strain VI and the unstable L-form 1 Ca/pen were 0·16 % and 0·4 % respectively (Martin, 1963*b*).

DISCUSSION

A paraphrase of Weidel's remark about bacterial cell walls (Weidel *et al.* 1963) would serve as the most appropriate characterization of the stable L-forms of *Proteus mirabilis:* 'An L-form is not an L-form, is not an L-form.' Conflicting reports about the presence or absence of mucopolymer components in stable L-forms (Weibull, 1958; Morrison & Weibull, 1962; Kandler & Zehender, 1957; Kandler *et al.* 1958) can be most easily understood by assuming that stable L-form strains with similar morphology but very different cell-wall structure were investigated in these cases. The available evidence strongly favours the view that stable L-forms of *P. mirabilis* constitute a heterogeneous group of organisms. Within this group the complex enzymic apparatus responsible for the synthesis of the various macromolecular components of the cell wall must be blocked at very different stages. Proteus L-form strains of the type of LD52, LVI, the type A and C strains of Kandler & Zehender (1957) and perhaps also strain L18, studied by Morrison & Weibull (1962), do not possess any mucopolymer. Our results suggest indeed that strain LD52 is completely devoid of any cell-wall material and thus represents a culture of true protoplasts which are able to multiply indefinitely, unlike the non-viable lysozyme protoplasts of Gram-positive bacteria (McQuillen, 1960).

Proteus L-form strain LVI seems to retain at least one of the plastic cell-wall layers, the lipopolysaccharide. This finding is not necessarily in conflict with Taubeneck's observation on the absence of bacteriophage receptors from strain LVI (Taubeneck, 1961). It is not known whether or not lipopolysaccharide acts as phage receptor in Proteus, as it does for the phages T3, T4 and T7 in *Escherichia coli* B. Moreover, the L-form lipopolysaccharide might have lost its receptor function as has happened with the lipopolysaccharide receptor analogue in the cell walls of phage-resistant *E. coli* B/3, 4, 7 (see Weidel, 1958).

Stable L-forms of the type of Proteus strains 1 Ca/20 and 1 Ca/25, which incorporate continuous but non-rigid mucopolymer basal structures in their cell walls, have not been studied extensively before. Chemical data on such mucopolymers do not offer support for the supposition that the mechanical defects are caused by a specific inability to synthesize any single mucopolymer constituent, as happens for instance in the diaminopimelic acid-less mutants of *Escherichia coli* (Baumann & Davis, 1957; McQuillen, 1958). Mucopolymers found in rod-shaped Proteus bacteria and in stable and unstable L-forms of Proteus are of a remarkably similar composition. Molar ratios of building blocks generally range around the values: muramic acid:glucosamine:diaminopimelic acid:glutamic acid: alanine = 1:1:1:1:1·7. Thus, Proteus mucopolymers closely resemble mucopolymers previously found in some other Gram-negative bacteria (Martin & Frank, 1962; Weidel *et al.* 1963). Another common feature of rigid layers in these enteric-bacteria is the covalent bonding of a protein component to the mucopolymer basal structure.

It is significant that mucopolymer-containing stable L-forms arise from the particular type of unstable L-form occurring in Proteus. In the latter organism the consequences of penicillin action observed in other bacteria, namely inhibition of mucopolymer synthesis (Park & Strominger, 1957) and autolytic removal of mucopolymer from cell walls (Weidel *et al.* 1960; Leutgeb *et al.* 1963), are definitely not operative. Here, the penicillin seems to act more specifically, presumably by preventing the formation of certain cross-linkages within the mucopolymer which are indispensable for the establishment of shape and mechanical stability. Studies on the nature and amount of some cross-linkages in different Proteus mucopolymers will be described later.

It remains for further studies to explore how the originally reversible lesions which penicillin induces in the mucopolymer of unstable L-forms of Proteus become perpetuated as hereditary defects in the mucopolymer-synthesizing stable L-form. In this type of L-form (which we propose to call 'spheroplast L-form', in contrast to the 'protoplast L-forms' which are essentially without cell walls) construction of the rigid layer must be blocked at a late stage, involving the morphogenesis of the R-layer rather than synthesis of its structural polymer. In this context the different ways of attachment of the protein component in the various R-layers to the mucopolymer basal structure, as seen in the electron microscope, should be noted. The typical surface pattern of protein granules is present only in rod-shaped rigid layers. No regular organization of the R-layer protein can be recognized in any of the non-rigid defective 'R-layers' of the L-forms. A useful working hypothesis for further studies might be to assume that the R-layer protein has an important function in the morphogenesis of the mucopolymer. In normal, bacillary organisms this function

might be closely related to the specific spatial arrangement of the protein within the R-layer complex. Disorganization of this arrangement brought about by the toxic action of penicillin or by the acquisition of hereditary structural defects of a yet unknown nature might render the R-layer protein incapable of carrying out its normal function of disposing the synthesized mucopolymer into a rigid rod-shaped basal structure.

Thanks are due to Professor O. Kandler for his continued support and encouragement.

This work was supported by a grant from the Deutsche Forschungsgemeinschaft. The author is greatly indebted to Dr P. H. Hofschneider and Miss Anneliese Preuss for their help in producing the electron micrographs. The technical assistance of Miss Karen Stermose and Mrs Renate Beck is gratefully acknowledged.

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Proteus mirabilis. Unstable L-form, strain 1 Ca/pen. Flagellated spherical cell covered with fimbriae and loosely attached superficial cell wall material. Magnification, × 14,000.

Fig. 2. Proteus mirabilis. Stable L-form, strain 1 Ca/20. Organisms with sharply defined surface, sparsely covered with slender fimbriae. Magnification, × 14,700.

Fig. 3. Proteus mirabilis. Unstable L-form, strain 1 Ca/pen. Empty cell wall after lysis with 0·4% (w/v) sodium dodecylsulphate. Magnification, × 16,800.

Fig. 4. Proteus mirabilis. Stable L-form, strain 1 Ca/20. Cell walls left after lysis with sodium dodecylsulphate. Magnification, × 16,800.

PLATE 2

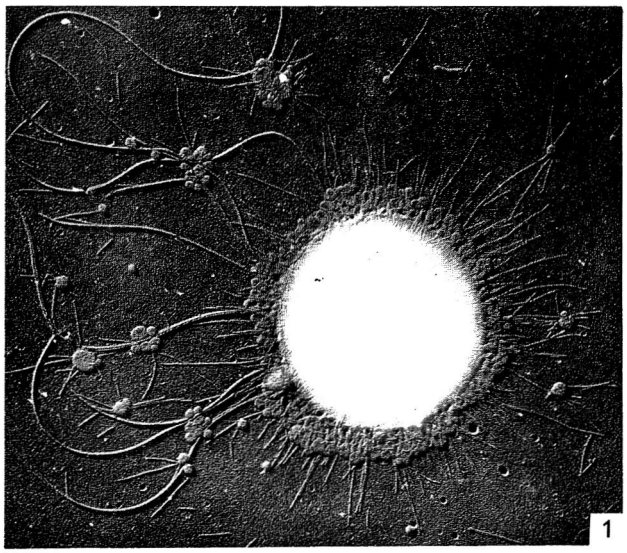
Fig. 5. Rod-shaped Proteus strain 1 Ca/5. Rigid layers, isolated from cell walls by extraction with phenol. Magnification, × 18,000.

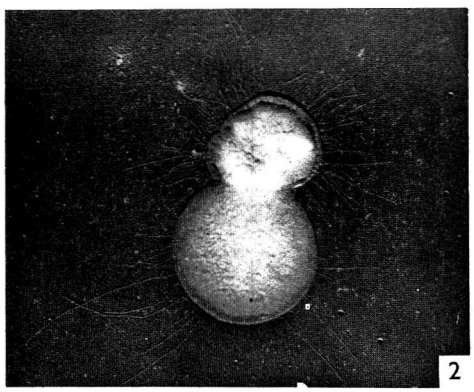
Fig. 6. As in fig. 5. Mucopolymer basal structure obtained by treatment of rigid layer with pepsin. Magnification, × 21,600.

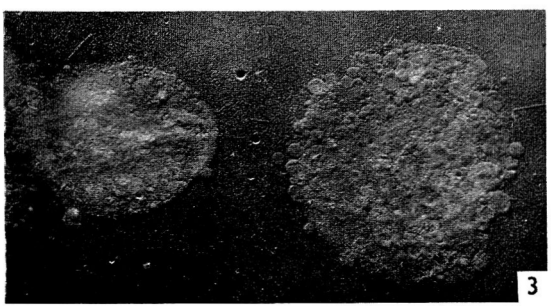
Fig. 7. Proteus mirabilis. Unstable L-form, strain 1 Ca/pen. Mucopolymer basal structures isolated from cell walls by extraction with phenol and treatment with trypsin. Magnification, × 18,000.

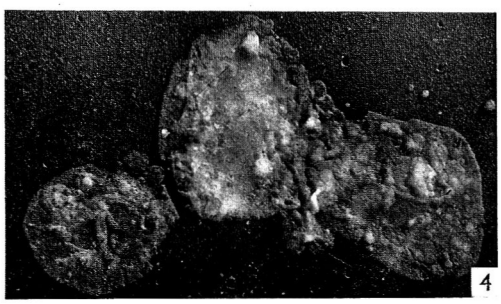
Fig. 8. Proteus mirabilis. Stable L-form, strain 1 Ca/20. Rigid-layer equivalent isolated from cell walls by phenol extraction. Magnification, × 21,600.

Fig. 9. As in fig. 8. Mucopolymer basal structure, obtained by trypsin treatment of R-layer equivalent. Magnification, × 21,600.









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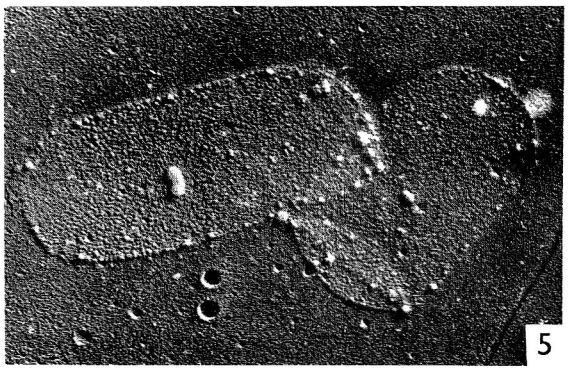

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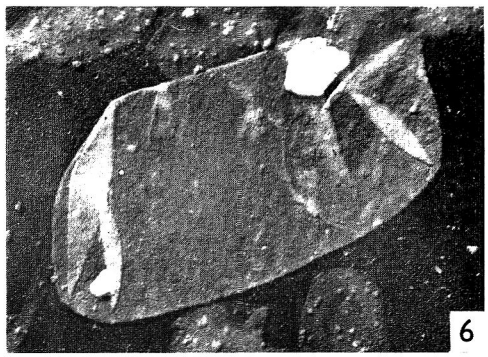

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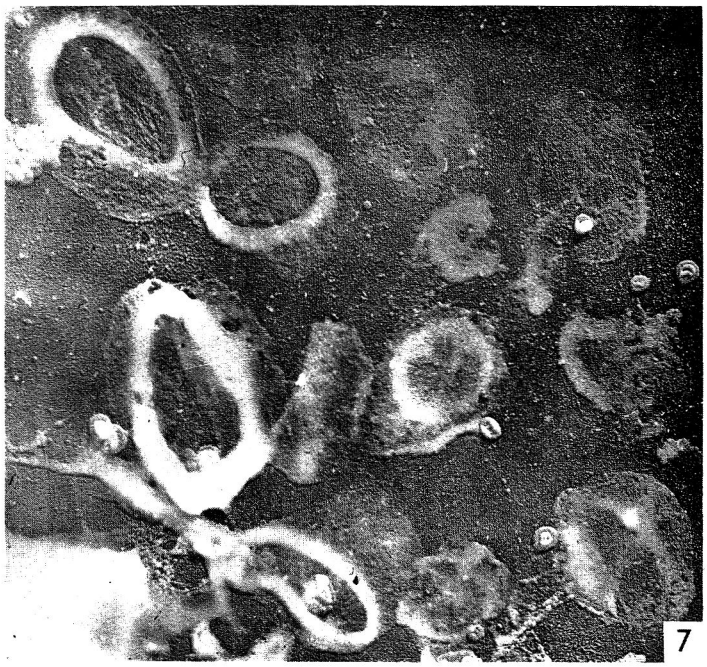

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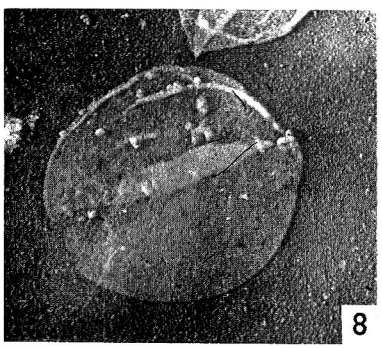

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J. gen. Microbiol. (1964), **36**, 451–459
Printed in Great Britain

Factors Influencing Osmotic Fragility of Mycoplasma

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(*Received 7 May* 1964)

SUMMARY

Osmotic lysis of Mycoplasma organisms was found to depend on the temperature of incubation. The organisms were practically resistant to osmotic shock at 0°, but lysed rapidly at 37°. Their sensitivity to lysis also depended on the pH value of the suspending medium, being lowest at pH values near neutrality. Divalent and polyvalent cations, in concentrations as low as 10^{-5} M, protected mycoplasmas from osmotic lysis. The parasitic Mycoplasma strains showed various degrees of osmotic fragility, *Mycoplasma gallisepticum* being the least fragile. All the parasitic strains tested were, however, more resistant to osmotic lysis than the saprophytic *Mycoplasma laidlawii*. The possibility that osmotic lysis of mycoplasmas involves autolytic processes was tested and is discussed.

INTRODUCTION

Mycoplasma organisms are limited by a thin membrane resembling the plasma membrane of bacteria, and are completely devoid of cell walls (Razin, 1963*a*). In spite of the absence of cell wall, mycoplasmas were found to be much more resistant to osmotic lysis than bacterial protoplasts and spheroplasts (Razin & Argaman, 1963). Further work showed that various Mycoplasma strains differed in osmotic fragility and that the saprophytic *Mycoplasma laidlawii* strains had a high degree of osmotic fragility when harvested at the logarithmic phase of growth (Razin, 1963*b*). To elucidate the mechanism of osmotic lysis of myeoplasmas, a study of the factors which influence osmotic fragility was undertaken.

METHODS

Organisms. *Mycoplasma mycoides* var. *mycoides* (PG 1), *M. mycoides* var. *capri* (PG 3), *M. agalactiae* (PG 2), *M. neurolyticum* (PG 28) and *M. gallisepticum* (PG 31) were obtained from Dr D. G. ff. Edward (The Wellcome Research Laboratories, Beckenham, Kent). A strain of *M. gallisepticum* named (R) was provided by Mrs Ruth Bernstein (Faculty of Agriculture, The Hebrew University, Rehovoth, Israel). Strain S 6 of *M. gallisepticum* was obtained from Dr H. E. Adler (School of Veterinary Medicine, University of California, Davis, U.S.A.). *Mycoplasma laidlawii* (oral strain) was isolated in our laboratory from the human oral cavity. This strain was serologically related to *M. laidlawii* strain A.

Suspensions of organisms. The organisms were grown in a modified Edward medium (Razin, 1963*b*) containing 1 % (v/v) Difco PPLO serum fraction. Growth was done in 500 ml. volumes distributed in 1 l. Roux flasks. The flasks were incubated

in inclined positions to improve aeration. The organisms were harvested while the optical density of cultures was still increasing, usually after incubation for 24–48 hr at 37°. The organisms were sedimented by centrifugation at 16,000 *g* for 5 min., washed twice in 0·25 M-NaCl solution and resuspended in the same solution.

Preparation of protoplasts. Protoplasts of *Micrococcus lysodeikticus* were prepared by lysozyme treatment, as described previously (Razin & Argaman, 1963) and suspended in a M-NaCl solution.

Examination of lysis. Test tubes contained 4 ml. of serial twofold dilutions of a 0·25 M-NaCl solution for testing the lysis of mycoplasmas, and of a M-NaCl solution for testing the lysis of protoplasts. The tubes were placed in a 37° water bath and each received 0·1 ml. of organism suspension. The number of viable particles in Mycoplasma suspensions was determined after 30 min. incubation according to Butler & Knight (1960). Simultaneously, the extinction of suspensions at 500 mμ was determined in a Unicam SP 500 spectrophotometer. The suspensions were then centrifuged at 20,000 *g* for 10 min., and the extinction of the clear supernatant fluids measured at 260 mμ. For testing the effect of temperature on osmotic lysis, extinction measurements were done in a Unicam SP 500 spectrophotometer equipped with a 'constant-temperature cell housing'.

Breakdown of cell membranes. Cell membranes of *Mycoplasma laidlawii* were prepared by osmotic shock of the organisms. Saline-washed packed organisms (1–2 g. wet weight) were suspended in de-ionized water (about 300 ml.), incubated at room temperature for 15 min. and then centrifuged at 16,000 *g* for 5 min. in the cold. The sediment, consisting mainly of unbroken organisms, was discarded and the supernatant fluid centrifuged at 34,000 *g* for 40 min. at 4°. Electron microscopy showed that this sediment consisted of membranes; no unbroken organisms were observed. The sedimented membranes were resuspended in ice-cold 0·01 M-tris buffer (pH 8·0) containing 0·01 M-NaCl. Proteolytic activity in membrane suspensions was tested by incubating 4 ml. samples of suspension at 37° for various periods of time. Extinction at 500 mμ of suspensions was measured at the end of the incubation period, and the membranes were sedimented by centrifugation at 34,000 *g* for 40 min. in the cold. The supernatant fluids were separated, concentrated by evaporation to 0·5 ml. volumes and their amino acid content analysed quantitatively by paper chromatography (Giri, Radhakrishnan & Vaidyanathan, 1952; Razin & Cohen, 1963). The coloured spots which appeared on the chromatogram after spraying with ninhydrin were cut off and eluted with 75 % (v/v) ethanol in water containing 0·005 % (w/v) $CuSO_4.5H_2O$. The colour intensity was determined spectrophotometrically at 540 mμ.

RESULTS

Effect of temperature on osmotic lysis

Lysis of *Mycoplasma laidlawii* by osmotic shock was temperature-dependent, and was not instantaneous; the time required for the completion of lysis depended on the incubation temperature (Fig. 1). Results obtained by the viable count technique confirmed those obtained by extinction measurements. The mycoplasmas were only slightly damaged by osmotic shock at 0° (Table 1). The dependence of osmotic lysis on temperature and incubation time was found also with *M. mycoides* var. *capri* and *M. neurolyticum*. The standard technique subsequently adopted for

testing osmotic lysis of mycoplasmas accordingly included incubation of suspensions in the hyptonic solutions at 37° for 30 min. Protoplasts of *Micrococcus lysodeikticus*, tested for comparison, lysed almost instantly in hypotonic NaCl solutions, the lytic process being even more pronounced at 0° than 18° (Fig. 2).

Resistance of heated Mycoplasma organisms to osmotic lysis

The degree of osmotic fragility decreased proportionally to the decrease in the number of viable organisms on heating Mycoplasma suspensions (Fig. 3). Heat-killed mycoplasmas were thus shown to be resistant to osmotic lysis while protoplasts of *Micrococcus lysodeikticus* heated to the same temperatures remained sensitive to osmotic lysis.

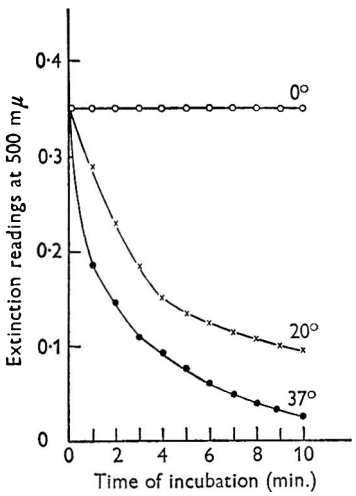

Fig. 1

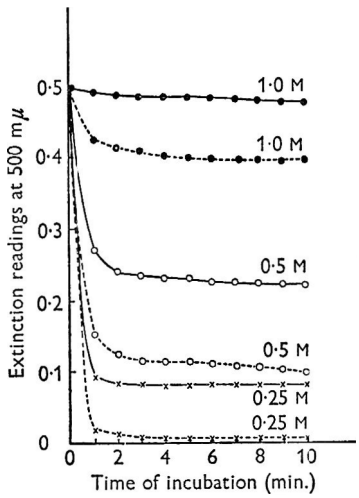

Fig. 2

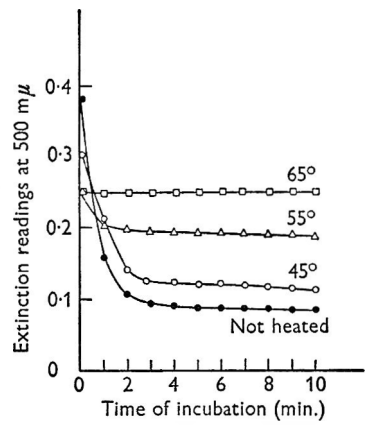

Fig. 3

Fig. 1. Osmotic lysis of Mycoplasma laidlawii at various temperatures of incubation. Test tubes containing 4 ml. de-ionized water were incubated at the temperatures indicated in figure. Each test tube received 0·1 ml. of a washed suspension of organisms in 0·25 M-NaCl and the extinction was measured at 1 min. intervals.

Fig. 2. Osmotic lysis of Micrococcus lysodeikticus protoplasts at various temperatures of incubation. Test tubes containing 4 ml. of various NaCl solutions (concentration of NaCl indicated in figure) received 0·1 ml. of suspension of washed protoplasts in M-NaCl. – – –, Suspensions incubated at 0°; ——, suspensions incubated at 18°.

Fig. 3. Osmotic lysis of Mycoplasma laidlawii organisms heated at various temperatures. Washed suspensions in 0·25 M-NaCl were heated for 10 min. at the temperature indicated in figure. Number of viable particles/ml. of unheated suspension, $1{\cdot}1 \times 10^{11}$; suspension heated at 45°, $1{\cdot}6 \times 10^{10}$; suspension heated at 55°, $5{\cdot}0 \times 10^{5}$; suspension heated at 65°, $2{\cdot}5 \times 10^{4}$. The heated suspensions were tested for osmotic fragility at 37°, as described in Fig. 1.

Effect of pH value on osmotic lysis

Sensitivity of *Mycoplasma laidlawii* to osmotic lysis varied with the pH value of the suspending medium. Sensitivity was lowest at values between pH 7·0 and 8·0 (Fig. 4). The mycoplasmas were somewhat more sensitive to lysis in dilute tris buffer than in phosphate buffer of the same pH value. The higher sensitivity of the mycoplasmas to osmotic lysis at acid or alkaline pH values was found also with *M. mycoides* var. *capri*.

Table 1. *Effect of temperature on viability of Mycoplasma laidlawii in various NaCl solutions*

Test tubes containing 4 ml. of NaCl solution or de-ionized water received 0·1 ml. of a washed suspension of organisms in 0·25 M-NaCl (final concentration of NaCl indicated in the table). Viable counts were made after 30 min. of incubation.

NaCl concentration (M)	Temperature of incubation		
	0°	18°	37°
	No. of viable organisms/ml. suspension		
0·250	$4·4 \times 10^9$	$4·5 \times 10^9$	$4·5 \times 10^9$
0·037	$3·6 \times 10^9$	$2·1 \times 10^9$	$7·0 \times 10^8$
0·006	$8·8 \times 10^8$	$1·1 \times 10^7$	$8·4 \times 10^5$

Table 2. *Protection by cations of Mycoplasma laidlawii and protoplasts of Micrococcus lysodeikticus from osmotic lysis*

Test tubes containing 4 ml. of neutralized solutions of the salts in de-ionized water received 0·1 ml. washed Mycoplasma suspension in 0·25 M-NaCl or washed protoplasts in 1·0 M-NaCl. Extinction of suspensions measured after 30 min. incubation at 37°.

Organism and Salt	Concentration of salt (M)					
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
	Extinction of suspension at 500 mμ					
Mycoplasma laidlawii						
Spermine.4HCl	—	0·42	0·38	0·37	0·37	0·08
Spermidine phosphate	—	0·41	0·40	0·35	0·20	0·08
$UO_2(NO_3)_2.6H_2O$	—	0·42	0·39	0·38	0·33	0·23
$AlCl_3.6H_2O$	—	0·37	0·36	0·36	0·36	0·30
$Fe_2(SO_4)_3$	—	0·45	0·44	0·40	0·39	0·30
$FeSO_4.7H_2O$	—	0·40	0·39	0·36	0·34	0·20
$MgCl_2.6H_2O$	0·43	0·40	0·40	0·36	0·33	0·20
$MnCl_2.4H_2O$	0·42	0·37	0·37	0·37	0·36	0·31
$CaCl_2$	0·47	0·39	0·35	0·34	0·18	0·09
$BaCl_2.2H_2O$	0·44	0·41	0·42	0·42	0·34	0·22
$CuCl_2.2H_2O$	0·45	0·39	0·37	0·37	0·36	0·17
$ZnCl_2$	—	0·44	0·40	0·40	0·39	0·30
$CdCl_2.2\frac{1}{2}H_2O$	0·41	0·39	0·39	0·38	0·35	0·30
$Hg(NO_3)_2$	—	0·42	0·38	0·38	0·31	0·13
NaCl	0·43	0·09	0·08	0·08	0·08	0·08
KCl	0·48	0·10	0·10	0·09	0·09	0·09
LiCl	0·39	0·08	0·08	0·08	0·07	0·07
NH_4Cl	0·39	0·10	0·08	0·08	0·08	0·08
Protoplasts of *M. lysodeikticus*						
Spermine.4 HCl	—	0·42	0·20	0·16	0·02	0·02
$MgCl_2.6H_2O$	0·27	0·08	0·08	0·07	0·02	0·02
$MnCl_2.4H_2O$	0·35	0·18	0·07	0·06	0·02	0·02
NaCl	0·04	0·02	0·02	0·02	0·02	0·02

Protective effect of cations

Trivalent and divalent cations were very effective in protecting *Mycoplasma laidlawii* against osmotic lysis; monovalent cations were completely ineffective. A long series of trivalent and divalent cations as well as spermine and spermidine inhibited osmotic lysis at 10^{-5} M or even lower concentrations (Table 2). Spermine prevented the death of *M. laidlawii* organisms in a highly hypotonic medium (Fig. 5). The polyamines and divalent cations afforded some protection against osmotic

lysis of *Micrococcus lysodeikticus* protoplasts, but at much higher concentrations than required to protect mycoplasmas (Table 2).

Osmotic lysis of parasitic Mycoplasma strains

In earlier studies parasitic Mycoplasma strains were found to be relatively resistant to osmotic lysis (Razin, 1963*b*). The finding that osmotic lysis of *Mycoplasma laidlawii* was temperature-dependent urged us to re-examine the osmotic fragility of the parasitic strains, by using 37° as the incubation temperature. In addition to measuring the extinction of suspensions viable counts were made. By these

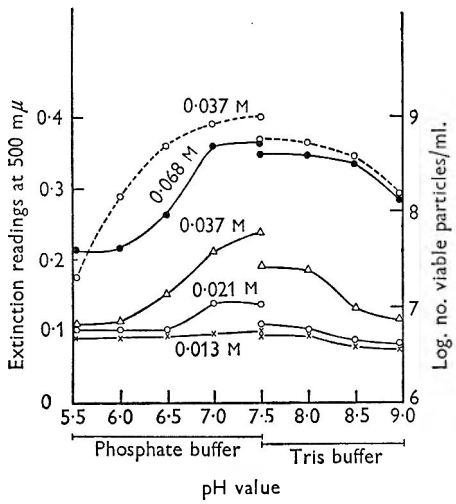

Fig. 4

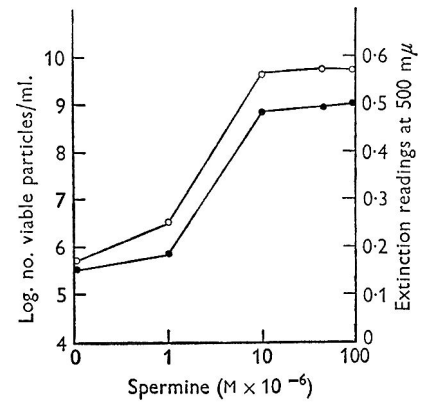

Fig. 5

Fig. 4. Osmotic lysis of Mycoplasma laidlawii at various pH values. Lysis was examined in NaCl solutions (concentrations indicated in figure) prepared in 0·01 M-phosphate buffer, or in 0·01 M-tris buffer. Extinction measurements (——) and viable counts (– – –) were made after incubation at 37° for 30 min.

Fig. 5. Protection by spermine against lysis and death of Mycoplasma laidlawii suspended in 0·006 M-NaCl. Viable counts (○) and turbidity measurements (●) were made after incubation at 37° for 30 min.

techniques the parasitic strains were found to lyse and die in hypotonic solutions (Fig. 6). However, the degree of lysis of the parasitic strains rarely reached that of the saprophytic *M. laidlawii* strains. The various parasitic strains differed in osmotic fragility. Thus, all three *M. gallisepticum* strains tested were usually more resistant to osmotic lysis than the other strains of parasitic Mycoplasma. The parasitic strains frequently showed variations in osmotic fragility. Apparently one of the factors responsible for these fluctuations is the age of the culture. This had already been demonstrated for *M. laidlawii* (Razin, 1963*b*) and was found to be true also for *M. mycoides* var. *capri*, which was much more sensitive to osmotic lysis when harvested at the logarithmic phase of growth (Fig. 7). On testing osmotic fragility of the parasitic strains, it was noted that the number of viable *M. neurolyticum* organisms significantly decreased during washing and incubation in 0·25 M-NaCl. The toxic effect of NaCl was less pronounced at lower temperatures. *Mycoplasma laidlawii*,

M. mycoides var. *mycoides* and *M. gallisepticum* were not significantly affected by 0·25 M-NaCl. Washing of *M. neurolyticum* in 0·4 M-sucrose + 0·01 M-phosphate buffer (pH 7·0) according to Rodwell & Abbot (1961) was found to be preferable to washing in 0·25 M-NaCl.

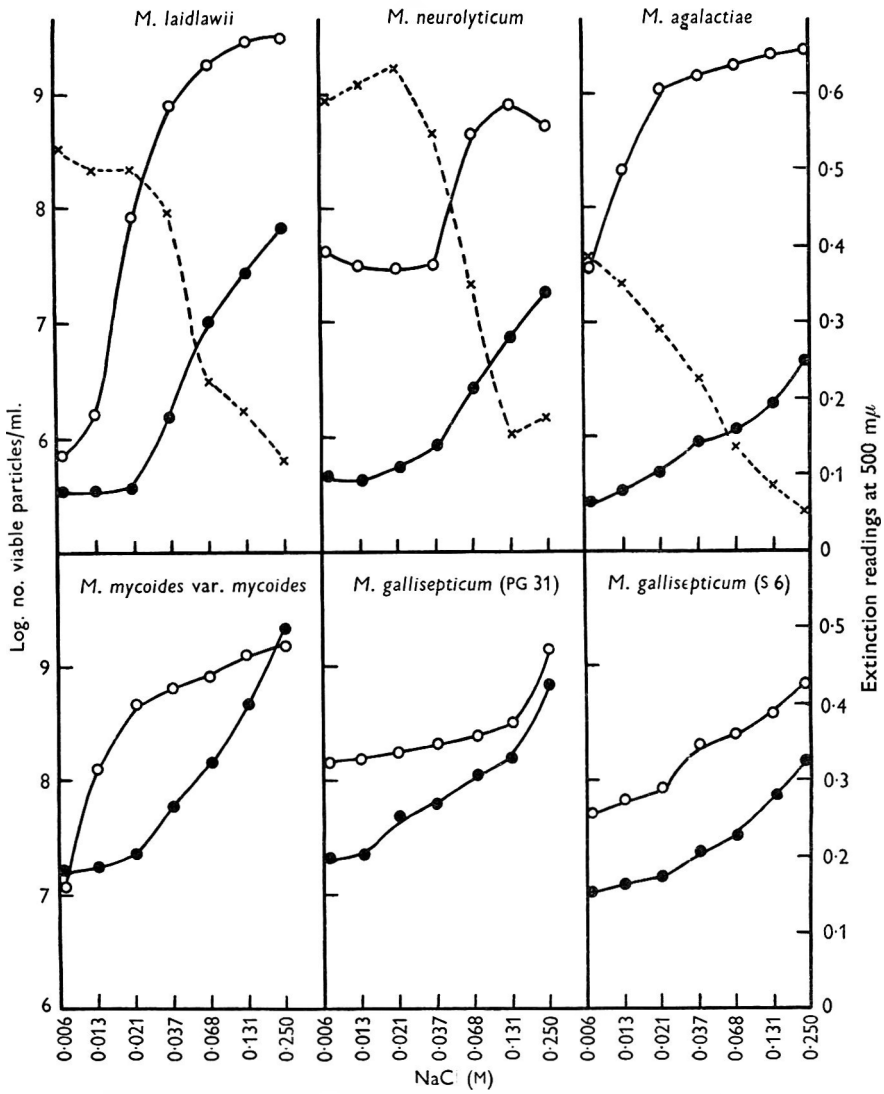

Fig. 6. Susceptibility of various Mycoplasma strains to osmotic lysis. The extent of lysis in the various NaCl solutions was measured after 30 min. incubation at 37°. Extinction readings at 500 mμ of suspensions (●); extinction readings at 260 mμ of the supernatant fluids of the corresponding suspensions (×); number of viable particles/ml. suspension (○).

Breakdown of Mycoplasma laidlawii membranes

Isolated membranes of *Mycoplasma laidlawii* were examined for disintegration by following the methods devised by Brown (1962). The optical extinction of membrane suspensions decreased markedly during the first 5 min. of incubation at 37°, followed

by an increase in the amount of free amino acids in the supernatant fluids of the suspensions (Fig. 8). However, electron microscopy revealed no gross morphological changes in the membranes, even after incubation at 37° for 1 hr.

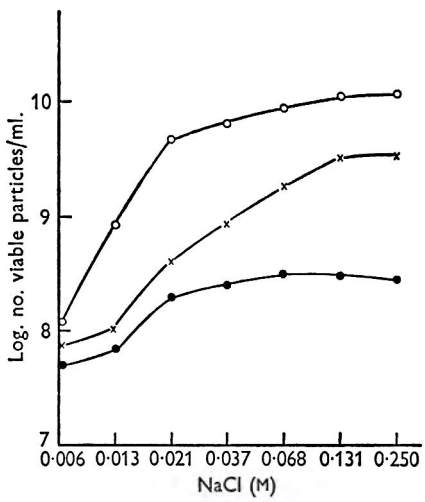

Fig. 7

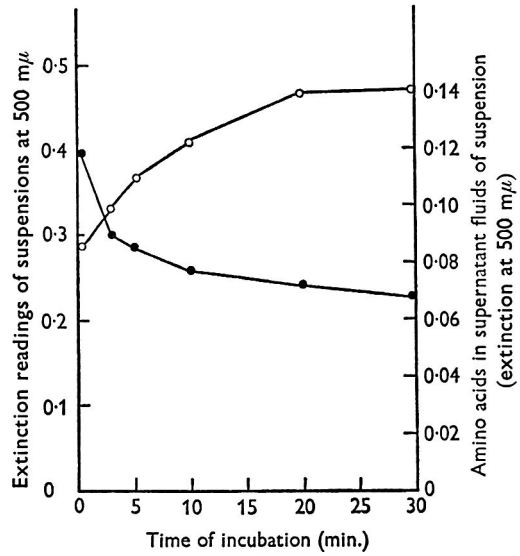

Fig. 8

Fig. 7. Death of Mycoplasma mycoides var. capri in hypotonic NaCl solutions. Organisms grown in Edward medium were harvested after 22 hr (○), 38 hr (×) or 66 hr (●) incubation at 37°. The washed organisms were suspended in the various NaCl solutions and viable counts made after 30 min. incubation at 37°.

Fig. 8. Proteolysis in suspensions of Mycoplasma laidlawii membranes. The membranes were incubated at 37° in 0·01 M-NaCl containing 0·01 M-tris buffer (pH 8·0). Extinction of suspensions (●) and the amount of free amino acids in the supernatant fluids of centrifuged suspensions (○) were determined after various incubation times as described under Methods.

DISCUSSION

The dependence of the osmotic lysis of Mycoplasma organisms on temperature may explain the observations of Butler & Knight (1960) that washed *Mycoplasma bovigenitalium* organisms rapidly died in 0·01 M-phosphate buffer at room temperature (about 20°), but survived well in the same hypotonic buffer at 2–4°. Several of the parasitic Mycoplasma strains previously found to be relatively resistant to osmotic lysis at room temperature (Razin, 1963*b*) were lysed at 37°. However, even then strains of parasitic Mycoplasma in general showed a higher resistance to osmotic shock than strains of saprophytic Mycoplasma. Most resistant to osmotic lysis were *M. gallisepticum* strains. This observation might be of some interest in view of the opinion that *M. gallisepticum* should not be regarded as Mycoplasma proper (Klieneberger-Nobel, 1963). However, electron microscopy (Ruys & Van Iterson, 1961) and chemical analysis of organisms (Morowitz *et al.* 1962; Razin, Argaman & Avigan, 1963) did not show any fundamental difference between *M. gallisepticum* and other strains of Mycoplasma. The lower osmotic fragility shown by several strains of parasitic Mycoplasma may in part be due to their being harvested at the decline phase of growth. Like saprophytic organisms (Razin, 1963*b*), parasitic

Mycoplasma organisms were also found to be most sensitive to osmotic shock when harvested in the logarithmic phase of growth. Harvesting of the more exacting Mycoplasma strains at this phase was not always possible, because of their poor and sluggish growth.

Inhibition of osmotic lysis of Mycoplasma fits in well with the known protective effect of di- and multi-valent cations on osmotically fragile organisms and organelles (Tabor, Tabor & Rosenthal, 1961). The ability of very low spermine concentrations to protect Mycoplasma organisms from lysis and death in highly hypotonic media at 37° may be of use in nutritional and metabolic studies, where viability and metabolic activity of the organisms can thus be kept under the above-mentioned conditions.

The temperature dependence of the osmotic lysis of Mycoplasma, and the resistance of heated mycoplasmas to lysis, suggested that the lytic process may involve autolytic enzyme activity. Autolysis might be triggered by initial damage to the cell membrane by osmotic shock. The studies of Brown and his collaborators on the lysis of marine and extremely halophilic bacteria provide certain hints in favour of this hypothesis. The envelope of the extremely halophile *Halobacterium halobium* resembles that of Mycoplasma in being composed of a lipoprotein unit membrane devoid of the mucopeptide polymer (Brown & Shorey, 1963; Brown, 1963). The envelope of a marine pseudomonad was composed of two lipoprotein membranes (Brown, Drummond & North, 1962). The membranes of the pseudomonad, when isolated by osmotic lysis of the organisms, underwent intense autolysis of a proteolytic nature (Brown, 1961). The autolytic process, like the osmotic lysis of Mycoplasma, depended on temperature, pH value and ionic strength of the medium, and was inhibited by di- and multi-valent cations (Brown, 1962). Our results indicate that disintegration and proteolysis of *Mycoplasma laidlawii* membranes might take place on incubation at 37°. However, gross disintegration like that described for membranes of the marine pseudomonad (Brown *et al.* 1962) could not be seen in the electron microscope. Autolytic processes therefore probably play only a secondary role in the clearing of Mycoplasma suspensions after the initial osmotic shock. The studies of Edebo (1961*a–c*) on sonic and osmotic lysis of bacteria may provide another approach to explain the effects of incubation temperature, heat, pH value and polyvalent cations on osmotic lysis. According to Edebo sonic or osmotic lysis of bacteria is a two-step process; the first step involving initial structural damage to the cell envelope, and the second, the dispersion of protoplasm in the surrounding medium. All the factors which influence sonic or osmotic lysis are known to affect the transformation of the protoplasmic gel into a sol, and its dispersion in the medium.

It is a pleasure to acknowledge the expert technical assistance of Mr M. Wormser. This investigation was supported in part by the Hebrew University Authority for Research and Development.

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The Significance of Bacteriophage in Bacterial Classification. A Review

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(*Received* 12 *May* 1964)

A given race of phage grows in a relatively limited range of bacteria. A coli phage, for instance, will not lyse a staphylococcus or a corynebacterium. Within these limits, however, some phages have a much wider host-range than others: some attack only one or a few bacterial strains; some a whole species; and some can lyse members of several species which on other grounds are considered to be not too distantly related. For instance, some pasteurella phages also attack strains of Salmonella and Shigella (Lazarus & Gunnison, 1947). The phage-sensitivity of a strain as a basis for bacterial classification can be interpreted in two ways, just as there are two levels at which bacterial classification can itself be regarded. That is to say, either as just another phenotypic character which the two strains may have in common; or at the level of the genetic material, the nucleic acid, so that, if two bacterial strains interact with the same phage at the genetic level, each of the strains is manifesting some degree of genetic compatibility with the phage, and thus with each other.

Receptors

To consider the first of these, one might argue that the phenotype indirectly portrays the genetic make-up of the strain, but we know, in fact, that it does this to a lesser extent than at first appears. Thus, although different kinds of organisms may produce enzymes with the same function, the actual enzyme proteins will probably differ structurally in different species (Markert & Møller, 1959; Signer, Torriani & Levinthal, 1961; Levinthal, Signer & Fetherolf, 1962). Nevertheless, the first step in phage-bacterial interaction being attachment of the phage particle to the surface of the cell, it is immediately apparent that the first necessary condition for sensitivity of a bacterium is that the receptor for the phage should be present, and accessible. The receptor may, with some phages, be part of the O somatic antigen, or, instead, part of the surface of a rough strain (Burnet, 1930). It may be a superficial envelope antigen, such as the Vi antigen (Craigie & Brandon, 1936), or it may even be provided by filamentous appendages such as fimbriae (Crawford & Gesteland, 1964), or flagella (Sertic & Boulgakov, 1936). Resistance of a bacterial culture arising from lack of attachment of the phage may sometimes be for only a trivial reason, for any manipulation or event, not necessarily genetic in origin, which does away with the receptor or prevents the phage particles having free access to it makes the bacterium resistant. There are innumerable examples of such a situation: a phage adsorbing to flagella will not attack bacteria growing at a temperature too high for flagella to be formed (Meynell, 1961); and phages for *Bacillus anthracis* will not attack bacteria

grown under conditions in which the capsule is produced, for the capsule obscures the receptor on the cell surface (Meynell, 1963). The example perhaps most often quoted is that of the strain of *Escherichia coli* which produces abundant Vi antigen at 20°, and at this temperature is sensitive to a Vi phage but resistant to another phage which uses the O somatic antigen as its receptor. When the bacteria are grown at 41·5°, however, they do not produce Vi antigen, and are now resistant to the Vi phage and sensitive to the O phage (Nicolle, Jude & Diverneau, 1953). A bacterium without an accessible phage receptor is thus in fact resistant to the phage, although it may be potentially sensitive, as has actually been demonstrated by infection of bacterial protoplasts by phage DNA (Spizizen, 1957) and the introduction of prophage by conjugation (Luria & Burrous, 1957). On the other hand, attachment or lack of attachment is not the only factor concerned in phage-sensitivity: certain staphylococcal phages adsorb to *Bacillus subtilis*, but produce no effect on the bacteria (Rakieten & Rakieten, 1937).

Killing without multiplication

In phage typing, the phages are used at 'routine test dilution' (Anderson & Williams, 1956) to distinguish the different bacterial strains, for it is the activity of the *bulk* of the phage population that is being tested, and its ability to *multiply*. If clearing occurs only at higher concentrations of phage, this can indicate either that the effect is due to host-range mutants of the typing phage or even to contaminant phage particles present in small numbers in the typing preparation (Rountree, 1956), or that, although the phage cannot multiply, it can nevertheless kill the bacteria, producing clearing like that caused by a colicine. Examples of this sort of killing include the lysis of Vi + *Salmonella typhi* by unadapted preparations of Vi phage II (Anderson & Fraser, 1955), and also the killing of Pseudomonas and Serratia species by coliphage P1 (Amati, 1962). The fact even that killing occurs must indicate some interaction of the phage with the bacteria, and a group of bacterial strains which are all killed by the same phage must, at least, share the receptors to adsorb it. But without knowing more about what the process of killing by phage consists of, we cannot put its significance in assessing bacterial relationships any higher than this.

Genetic similarity. Nature of phage

Before we look for any relationship between a particular phage and bacterium at the genetic level, we must first ask if it has any meaning at all to talk of a fundamental, natural relationship between phage and its host. We must, that is, consider the essence of the relationship between phage, as a biological entity, and bacterium; or, put another way, what is the fundamental nature of the phage? During the lytic cycle, the phage is a parasite, multiplying autonomously. The phage genome controls its own replication, and directs the synthesis of its protein—protein foreign to the bacterial cell. In the lysogenic state, the phage is an integral part of the bacterial heredity; the phage genome is replicated under the same control as the bacterial genome itself (Nagata, 1963), it segregates with it, and is evidently physically associated with it, in some cases at least. The more we learn about the structure and behaviour of phage, the more probable it becomes that the phage originated as a genetic element of the bacterium, and evolved by developing the means for cell-to-

cell transfer. This was first proposed because of the abundance of lysogenic strains and the enormous variety of different phages specifically attacking different races of bacteria (Nicolle, Grabar & Gilbert, 1946; Felix, 1953). Certain phenomena also suggest that genetic exchange can actually occur between phage and host bacterium. For instance, with several different phages, it has been observed that when ultraviolet-irradiated phage is grown in bacteria which have also been irradiated, new genotypes appear which are different from the phage mutants obtained in other circumstances (Jacob, 1954; Tessman & Ozaki, 1960), and which are best explained as the result of genetic exchanges between phage and host genomes occurring with increased frequency as a result of the ultraviolet irradiation (Roman & Jacob, 1957). Some of these new genotypes, formally similar to mutants, are, in fact, apparently mutated at several distinct loci simultaneously, and different kinds are obtained when the phage is grown in different bacterial hosts (Fraser, 1957).

Lysogeny, which involves integration of the phage genome with the bacterial genome implies some degree of genetic homology between them. A genetic map based on recombination frequencies can be made for phage, just as for bacteria, and the phage chromosome, like that of the bacterium, appears to be a circular structure (Jacob & Wollman, 1961; Cairns, 1963; Foss & Stahl, 1963; Epstein *et al.* 1963; Chandler, Hayashi, Hayashi & Spiegelman, 1964). A short region of the genome of a temperate phage is concerned with the ability to lysogenize (Kaiser, 1957; Levine, 1957), and integration of the prophage has been visualized as first involving pairing of this region with a homologous region of the bacterial chromosome. In one mechanism which has been proposed (Campbell, 1962), the pairing of the homologous regions of the two circular structures is followed by a cross-over between them which leads to insertion of the phage genome, opened into a linear structure, into the bacterial genome between the ends of the break where the cross-over took place.

Some phages, such as coliphage λ, have a fixed location for their prophage, while the prophages of other phages may attach at one or other of a few, or even sometimes of many different genetic sites (Lederberg & Lederberg, 1953; Jacob & Wollman, 1957; Bertani, 1958). This immediately presents an analogy with the bacterial fertility factor, F, which in the ordinary way can be integrated anywhere on the bacterial chromosome, but whose site becomes fixed after picking up bacterial genes, like those concerned with lactose fermentation, for then it is determined by the homology of those associated genes with their alleles on the bacterial chromosome (Adelberg & Burns, 1960). The analogy between phage and the bacterial fertility factor can be pressed further, for the λ prophage can acquire bacterial genes located next to it. When the prophage is induced, the resulting phage particles which emerge contain an incomplete phage genome and less than the full complement of phage DNA and carry the bacterial markers into their next host (Arber, 1958; Campbell, 1959; Weigle, Meselson & Paigen, 1959). And again, the bacterial markers on entering the new host may occasionally be integrated into the bacterial genome, or else may remain associated with the carrier genetic fragment, the defective prophage, just as they may remain associated with F, so that there appears to be no fundamental difference between these two genetic elements.

Similarities at the genetic level between one bacterium and another have been assessed by the readiness with which genetic interchange occurs between them as well as by physico-chemical tests to compare the base compositions and base

sequences of their nucleic acids (Marmur, Falkow & Mandel, 1963). The overall composition of a sample of DNA, usually denoted by its content of guanine-cytosine pairs, determines its density and also the temperature to which it has to be heated to denature it by separating the two strands of the helix (Sueoka, Marmur & Doty, 1959; Marmur & Doty, 1959). The absolute sequence in which the nucleotides are joined in a DNA strand has been investigated for a few micro-organisms by the 'nearest-neighbour' method; that is, the relative frequencies with which each nucleotide is situated next to itself or to each of the others (Josse, Kaiser & Kornberg, 1961). Finally, the base sequences in nucleic acids from different sources have been compared, using RNA or single strands of DNA. Reforming of the DNA duplex structure or fixing of RNA to DNA needs matching of complementary regions in the individual strands, and so the readiness with which hybrid combinations are formed gives an estimate of the extent to which the nucleic acids are alike in base sequence (Schildkraut, Marmur & Doty, 1961). We are all aware of the agreement between genetic experiments and the results of these physico-chemical tests on the molecular structure of the nucleic acid. We know that a genetic fragment donated by one bacterium to another may or may not become integrated into the recipient's chromosome, and that, broadly, the less similar the DNAs of the two bacterial strains, the less likely is integration to occur. For instance, integration seldom occurs between *Escherichia coli* and Salmonella species, which, although they have similar overall base compositions and the same gross ordering of genes on the chromosome, show only localized regions of base sequence homology in physico-chemical experiments (Falkow, Rownd & Baron, 1962).

I can find the base compositions of only a very few phages reported in the literature (Lanni, 1960; Erikson & Szybalski, 1964), but it is held that lysogenization occurs only when the DNA of the phage has the same overall composition as that of the bacterium. Integration requires a base sequence homology sufficient to lead to pairing, but one might imagine that in the integration of prophage, only a very localized homology might be needed, and thus that overall similarity in base composition between bacterial and phage DNA might not necessarily be observed. However, we know that the DNA from any single bacterial source is relatively homogeneous in composition for, in contrast to the enormous differences between different microbial DNAs, no gross local differences in guanine-cytosine content along one single kind of DNA appear when it is broken down into smaller pieces (Marmur & Doty, 1959; Sueoka *et al.* 1959). It has been calculated that there can be few, if any, sizeable DNA fragments in common between organisms with overall base compositions differing by 10 %, or perhaps even less, and it would therefore not be surprising if a bacterium were lysogenized only by a phage whose DNA was similar to its own. Incidentally, phage λ and *Escherichia coli*, which have both been examined for their base sequence, have also shown the same nearest-neighbour frequencies (Josse *et al.* 1961); and a comparison of their base sequences by combination of nucleic acid strands has led to the conclusion that about 8 % of the λ genome is complementary to *E. coli* DNA (Green, 1963).

Significance of lytic multiplication

Foreign bacterial genes in a fragment of genome too dissimilar to be integrated in the recipient can often function nevertheless, and also be replicated if they are

associated with a structure, such as the F factor, itself able to replicate autonomously. Examples of this are the substituted F factor from *Escherichia coli* when it is in *Salmonella typhi*, or even in Proteus, Vibrio, Serratia or *Pasteurella pestis* (Mäkelä, Lederberg & Lederberg, 1962; Falkow, Wohlhieter, Citarella & Baron, 1963; Marmur *et al.* 1961; Martin & Jacob, 1962). The cell can interpret the foreign code and make the enzyme protein characteristic of the donor cell which contributed the genetic fragment (Signer *et al.* 1961; Levinthal *et al.* 1962). By analogy with such a situation, one might expect to find temperate phages (that is, phages inherently capable of lysogenizing) which could multiply autonomously, that is, by the lytic cycle, in many more different kinds of bacteria than they could lysogenize. I can find no instance of exactly this in the literature, but we know, of course, that phages which never lysogenize, like the T even-T5 phages, can multiply in bacteria with a base composition very different from their own (Lanni, 1960; Erikson & Szybalski, 1964). We can also conclude, since one and the same phage can attack bacteria as different as *Escherichia coli* and *Pasteurella pestis*, that there must be many more cases besides the T even-T5 phages where similarity in nucleic acid composition is not needed for the phage to multiply. Thus multiplication does not necessarily imply close genetic relationship of the phage with its host.

Significance of transduction

Similarly, a phage can incorporate in its protein coat foreign DNA different in composition from its own. A *Bacillus subtilis* phage which differed from its host in DNA composition was nevertheless found to be able to transduce a variety of bacterial characters. The transduced markers, in the form of DNA with the characteristic bacterial composition, were, however, a physically separate element from the phage DNA (Okubo, Stodolsky, Bott & Strauss, 1963). This finding brings out the difference between specialized and generalized transduction as indicators of similarity at the genetic level. Specialized transduction (such as occurs with phage λ), where the markers are situated next to the location of the prophage and are physically associated with a defective phage genome, probably signifies a degree of homology and fundamental relationship between phage and host. But the same cannot be said about generalized transduction, where any different bacterial character is picked up by the phage in the course of lytic multiplication, and is evidently free of any residual phage genome when it arrives in the new host.

Application of phage sensitivity

It is by now apparent that many factors are involved in phage-bacterium interactions and in the sensitivity of a bacterium to a phage. One question here is how do they apply directly to the application of phage sensitivity in bacterial classification and phage-typing? First, the bacterium may be resistant because it does not adsorb the phage. Between members of larger taxonomic groups the specificity lies largely in adsorption, but one would infer that if adsorption were to take place, one would then see the patterns of behaviour resulting from genetic incompatibility. The main use to which phage-typing is put is to distinguish between very closely similar bacterial strains which cannot be distinguished in other ways, and which are thus likely to be closely related. In the two most widely used typing schemes, those for *Salmonella typhi* and *Staphylococcus aureus* (Anderson & Williams, 1956), all the

strains likely to come under test adsorb all the typing phages, and failure of lysis is due to causes other than non-adsorption. One of these causes is pre-existent lysogenicity of the bacteria with a phage like the typing phage in any scheme using a diversity of different phages for typing (Rountree, 1956). In this situation, of course, it would paradoxically be an absence of reaction with the typing phage that would be significant of a relationship. The presence of the phage genome in the cell is known to be able to affect it in a number of different ways, and two of these may influence its phage-sensitivity. First, the prophage directly renders the cell immune to lytic multiplication of similar phage, and, secondly, an alteration in the cell surface may occur resulting in antigenic change associated with loss of ability to adsorb one phage and perhaps gain of ability to adsorb another (Uetake, Luria & Burrous, 1958).

Finally, I must mention what in this context is perhaps the most subtle relationship of all between phage and host, that is, the phenomenon of host-induced modification (Bertani & Weigle, 1953). This is very much in evidence in the well-known typing scheme for *Salmonella typhi*, in which, as we know, a single phage is used which is altered by growth in different strains, so that it becomes 'adapted' to the strain in which it was last grown. In some cases, the adaptation consists of a mutation or hereditary alteration in the phage, but in others the change is a modification imposed by the last host on the crop of phage to issue directly from it (Anderson & Fraser, 1956). When host-induced modification occurs, a phage which has reproduced in one host is modified in a way that affects its ability to grow in another. Thus, a bacterial strain under test can react differently, not only with different phages, but actually with the same phage grown in itself or in a different host. The modification lies in the DNA of the phage and is such that, while this DNA is not attacked by the DNase of the bacterial strain in which it was made, it is extensively broken down on entering the new strain (Arber & Dussoix, 1962; Dussoix & Arber, 1962; Arber, Hattman & Dussoix, 1963). In this situation, the second strain will equally reject bacterial DNA of the first in the form of its chromosomal genetic markers (Arber, 1962), as well as extrachromosomal DNA structures like the F factor (Glover, Schell, Symonds & Stacey, 1963). Enzymes concerned with DNA, for instance methylating enzymes, are known to distinguish their homologous from heterologous nucleic acid (Gold, Hurwitz & Anders, 1963), so that we can well think of this phenomenon as another example of the recognition of 'self' in nature. But it is interesting to find that it applies to foreign DNA like phage, so that even when the DNA has a foreign code, the bacterium puts the same superficial imprint on its structure as it puts on its own.

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