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THE JOURNAL OF GENERAL MICROBIOLOGY

The *Journal* will publish accounts of original research in general microbiology, i.e. the study of bacteria, microfungi, microscopic algae, protozoa, and viruses in their biological activities and, more particularly, the fundamental aspects of the study of these forms, including structure, development, physiology, genetics, cytology, systematics and ecology. Writers of papers on a specialized aspect of their subject should describe their work so that its relevance to their own science and to microbiology in general will be apparent to readers who may be unfamiliar with the particular aspect.

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'Easy reading's curst hard writing.'—*The Editors, J. gen. Microbiol.*

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Introduction; (*c*) Methods; (*d*) Results (illustrative protocols only should be included); (*e*) Discussion (if any), and general conclusions; (*f*) Acknowledgements; (*g*) References.

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PLANT PATHOGENIC FUNGI AND PLANT DISEASES. *List of Common British Plant Diseases*, 1944. (Cambridge University Press.)

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Incorporation of Amino Acid into Protein by Utilizing a Cell-Free System from Paramecium

By A. H. REISNER AND HELEN MACINDOE
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(*Accepted for publication* 14 *October* 1966)

SUMMARY

Cell-free incorporation of amino acid into paramecium protein was accomplished by using ribosomes, soluble fraction, guanosine triphosphate and adenosine triphosphate. Less than 20% of the incorporated label was detected in the soluble fraction, indicating that little if any complete *de novo* synthesis occurred. Incorporation was markedly decreased by submicrogram concentrations of RNase and by decreasing [Mg^{2+}] to below 3 μmoles/ml. A 'pH 5 fraction' from mouse liver was able to replace the paramecium soluble fraction but attempts to obtain active 'pH 5 fractions' from paramecium failed. Evidence is presented for the presence of polyribosomes in paramecium; there was some indication that they were active in amino acid incorporation. Following incorporation, 80s ribosomes labelled with amino acid were recovered; subjecting these to Mg^{2+}-deficient buffer caused dissociation into 45s and 30s units. A considerable portion of the label remained with the heavier unit.

INTRODUCTION

Fruton (1963) and Campbell (1965) have recently reviewed the considerable research done in recent years on cell-free incorporation of amino acid into protein ('protein synthesis'). However, while extensive studies have been undertaken with bacterial and mammalian systems and to a lesser extent with extracts from fungal and higher plant tissue, work on the protozoa is almost non-existent. The literature contains three papers on *Tetrahymena pyriformis* (Mager & Lipmann, 1958; Mager, 1960; Seaman, 1962), and one on the trypanosome *Crithidia oncopelti* (Chesters, 1966). With respect to ribosomally-controlled protein synthesis only the studies of Mager & Lipmann and of Chesters are relevant. The present paper deals with amino acid incorporation into protein in a cell-free system obtained from the ciliated protozoan *Paramecium aurelia*. Because paramecium is genetically the most extensively studied protozoan, it seemed advisable to determine whether or not its protein synthesis fell into the 'classical' pattern.

METHODS

Adenosine triphosphate (ATP), guanosine triphosphate (GTP), creatine phosphate, creatine phosphokinase, pronase and ^{14}C-labelled amino acids were purchased from Calbiochem, Los Angeles, U.S.A. Reconstituted ^{14}C-protein hydrolysate (lot 6601) was obtained from Schwarz Bioresearch, Orangeburg, New York. Deoxyribonuclease (DNase) and ribonuclease (RNase) were obtained from Sigma Chemical Company, St Louis, Mo. Proteose peptone, yeast extract, Sabouraud dextrose broth and fluid

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thioglycollate medium were obtained from Difco Laboratories, Detroit, Michigan, Vegemite from Kraft Foods Ltd., Melbourne, Australia, and compressed bakers' yeast from the Yeast Company of Australia Pty. Ltd., Sydney. Lactalbumin hydrolysate (enzymic) was obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio, as was puromycin dihydrochloride. Streptomycin sulphate was obtained from Evans Medical (Pty.) Ltd., and chloramphenicol from Parke Davis and Co. Ltd., Sydney.

Organism. Paramecium aurelia stock 51 (syngen 4) free of κ and expressing serotypic antigen 51 A was used throughout the work. A culture of sterile organisms was kindly supplied to us by Dr W. J. van Wagtendonk and has now been maintained in our laboratory for 3 years.

Culture medium. Cultures were grown at 25° in a medium consisting of 0·25% (w/v) Vegemite, 0·2% (w/v) yeast extract, 0·75% (w/v) proteose peptone and 23% (v/v) dialysed yeast supernatant (see below). This medium was autoclaved as one mixture at 17 lb./sq.in. (about 123°) for 40 min. Growth rate was one fission/day and peak population equalled 4×10^3 organisms/ml.

Preparation of dialysed yeast supernatant fluid. To every 1000 ml. water was added 3·6 kg. of grated live compressed baker's yeast. The slurry was autoclaved for 45 min. at 10 lb./sq.in., cooled to room temperature and then centrifuged at 3000*g* for 15 min. The autolysate supernatant fluid was decanted into dialysis tubing and the tubing sealed air-free but with about 20% excess potential volume to preclude bursting. After autoclaving for 30 min., the material was dialysed for 60 hr against running tap water. The dialysis residue, when not used immediately, was either stored at 2° in the dialysis bags, or frozen and stored at −20°. It contained about 2% (w/v) solids.

Tests for sterility. All stock tubes of paramecia were routinely tested for bacterial sterility at the time of subculturing (once each week) by using Sabouraud dextrose broth, fluid thioglycollate medium and a yeast extract + lactalbumin hydrolysate medium (0·5% yeast extract, 0·5% lactalbumin hydrolysate, 0·05% glucose; w/v) and tests were observed for 7 days. From time to time sterility tests were held for 21–28 days as a further check on contamination by slow growing bacteria and fungi. Absence of observable growth in these tests was taken to mean that we were dealing with a single-membered (axenic) culture of paramecia.

Bacterial counts of experimental fractions were made by plating on 2% agar plates containing 0·5% (w/v) each of yeast extract, lactalbumin hydrolysate and glucose. Samples were also taken from each flask of a mass culture for testing in the three liquid media.

Preparation of cell fractions (all steps at 0–4°). Mass cultures of paramecia were harvested as previously described (Macindoe & Reisner, 1967). Following packing of the organisms at 3000*g* the dark top layer of deposit was aspirated off. The organisms, after being resuspended in medium A (250 mM-sucrose, 50 mM-tris, 25 mM-KCl, 5 mM-$MgCl_2$, pH 7·6) at 5 to 7×10^6 organisms/ml., were homogenized manually in a glass and Teflon grinder. Less than 1% of whole paramecia were detected following this homogenization. The homogenate was centrifuged at 3000*g* for 15 min. and the supernatant fluid decanted and centrifuged at 10,000*g* for 15 min. This supernatant fluid was used for incorporation experiments not requiring additional fractionation. To separate ribosomes from the cell sap a 10,000*g* supernatant fluid was centrifuged for 40 min. at 150,000*g*. The top two-thirds of this supernatant fluid was removed for experimental use, the bottom third discarded, and after surface rinsing the pellet was resuspended in medium A.

Fractionation of mouse liver (all steps at 0–4°). Livers from freshly killed random bred mice were excised into cold medium A and minced. Additional medium A was added to give 2·5 ml. medium A/g. wet weight liver. The material was then processed as described in the previous section except that the 10,000g centrifugation was replaced by one at 12,500g.

Preparation of 'pH 5 fraction'. Freshly prepared 150,000g supernatant fluid from mouse liver was diluted threefold with medium A and placed in ice. With constant stirring it was adjusted to pH 5·2 by the dropwise addition of cold 0·1 N-acetic acid and then centrifuged for 15 min. at 3000g. After discarding the supernatant fluid the pellet was surface-rinsed with medium A and then resuspended in medium A and centrifuged again at 3000g for 15 min.; the supernatant was then decanted and held in ice until used.

Repeated attempts to obtain an active 'pH 5 fraction' from paramecia have failed.

Preparation of ^{14}C-amino acid-labelled 80s ribosomes. To prepare 80s ribosomes labelled with a single ^{14}C-amino acid the 150,000g pellet fraction from paramecia was obtained as described above. After resuspension in medium A from which sucrose was omitted it was mixed with 'pH 5 fraction' from mouse liver. The incubation conditions are given in Fig. 6*a*. Preparation of 80s ribosomes labelled with ^{14}C-reconstituted protein hydrolysate was done by using paramecium 10,000g supernatant fluid. Following incubation both types of preparation were layered on 15–30 % linear sucrose gradients in medium A and centrifuged for 3 hr at 53,000g in a Spinco SW 25-1 rotor. Samples (1 ml.) were collected through the bottom of the tubes, the E_{260} value determined and the samples containing the 80s particles pooled.

Chemical estimations. Protein was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951). Mg^{2+} was estimated by the Orange & Rhein (1951) method.

Radioactive counting. TCA insoluble material. Samples for counting were suspended in 5 % (w/v) TCA, heated 20 min. at 90°, chilled in ice and then centrifuged. The pellet was dissolved in 0·5 N-NaOH and then re-precipitated by adding two volumes of 10 % (w/v) TCA solution and centrifuged. The pellet was then resuspended and centrifuged first in water, then in ethanol and finally in ether. The ether-washed pellet was dissolved in 0·5 ml. Hyamine-10x to which was added 10 ml. scintillator (0·5 %, w/v, 2,5-diphenyloxazole (PPO) and 0·03 % w/v, 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene-(dimethyl POPOP) in toluene).

TCA soluble material. One ml. of aqueous sample (5 %, w/v, TCA) was added to 10 ml. of scintillation fluid (as above but with 2 % (v/v) Triton X-100 added). 0·6 g. of Cab-O-Sil (Godfrey L. Cabot. Inc., U.S.A.) was then added to the vial and the mixture shaken vigorously.

Counting was performed in an automatic liquid scintillation counter. Counting efficiency for ^{14}C based on internal standardization was 85 ± 2 % for all TCA insoluble samples and 67 ± 1 % for TCA-soluble samples.

Pronase-treated subfractions from paramecium 10,000g supernatant fluid. A paramecium 10,000g supernatant fluid was obtained and incubated as described in Table 3. Following incubation 9·8 ml. of the mixture (equiv. 49 mg. protein) was chilled to 0° and then centrifuged for 90 min. at 150,000g. The top 6·5 ml. of this 150,000g supernatant fluid were removed, mixed with an equal volume of saturated $(NH_4)_2SO_4$ solution and centrifuged for 20 min. at 14,000g. The pellet was dissolved in 2·5 ml. medium A (lacking sucrose) and dialysed overnight against two 1 l. changes of

medium. The remaining 150,000g supernatant fluid was discarded and the pellet surface-rinsed with medium A. After resuspending it in 2·5 ml. medium A (no sucrose) it was dialysed overnight against two 1 l. changes of the medium.

Following dialysis 0·2 mg. pronase was added to 1 ml. samples of the fractions which were then incubated for 45 min. at 37°, together with their controls. After adding 1 ml. of 10% (w/v) TCA the mixtures were centrifuged at 3000g and 1 ml. of each supernatant fluid was removed for counting. The remaining supernatant fluid was discarded and the TCA-precipitate worked up for counting.

RESULTS

Table 1 shows that the components of the 10,000g supernatant fluid were capable of incorporating amino acid. Both the 150,000g pellet and the 150,000g supernatant fluid were necessary as well as ATP, GTP, creatine phosphate and creatine phosphokinase (AGCC) as a group. In the complete system 1240 C.P.M./mg. pellet-protein were incorporated, the equivalent of $6{\cdot}7 \times 10^{-5}$ μmole valine. Comparable results were obtained in experiments with L-alanine-1-^{14}C and L-leucine-1-^{14}C.

Table 1. *Incorporation of L-valine-1-^{14}C into the components of paramecium 10,000g supernatant fluid fraction*

The 10,000g supernatant fluid was centrifuged 40 min. at 150,000g. Experimental conditions: volume = 1·7 ml. Label, L-valine-1-^{14}C 0·25 μc. (specific activity, 9·8 μc./μmole). AGCC = ATP, 5 μmoles; GTP, 0·5 μmole; creatine phosphate, 30 μmoles; creatine phosphokinase, 100 μg. 150,000g pellet, 190 μg. protein; 150,000g supernatant fluid, 600 μg. protein. Sucrose, 300 μmoles; tris, 60 μmoles; KCl, 30 μmoles; $MgCl_2$, 6 μmoles, pH 7·6. Incubated 45 min. at 33°, reaction stopped with addition of 2 ml. cold medium A containing 5 mg. bovine gamma globulin and 5 mg. of ^{12}C-DL-valine followed by 4 ml. 10% (w/v) TCA.

	Counts per min.	
	+AGCC	−AGCC
150,000g pellet + 150,000g supernatant	235	5
150,000g pellet	20	8
150,000g supernatant	5	6

The effect of various additives on incorporation of amino acid into a 10,000g supernatant fluid

The response of a paramecium 10,000g supernatant fluid to ATP, GTP, creatine phosphate and creatine phosphokinase in all admixtures is depicted in Fig. 1, as well as the effect of deoxyribonuclease and ribonuclease when added just before incubation. It is apparent that the DNase had little effect on incorporation (90 C.P.M. *vs.* 108) while RNase at a final concentration of 0·6 μg./ml. caused a marked depression of incorporation (31 C.P.M. *vs.* 108). Both GTP and ATP (or the ATP generating system) were required for maximum incorporation, but the ATP preparation also exerted inhibition. For example, when added to the other three co-factors, it caused a 40% decrease in incorporation.

The dependence of amino acid incorporation by the 10,000g supernatant fluid on [Mg^{2+}] is shown in Fig. 2. The concentration of Mg^{2+} was critical; maximum incorporation occurred at about 5 μmoles/ml.

Incorporation of amino acid into the 10,000g supernatant versus time

A time-course study of incorporation into a paramecium 10,000*g* supernatant fluid is shown in Fig. 3. Linear incorporation lasted less than 5 min. and no significant incorporation occurred after 10 min. The fact that no loss of TCA-insoluble label occurred up to 60 min. of incubation indicated that proteolytic activity was not significant under the incubation conditions used.

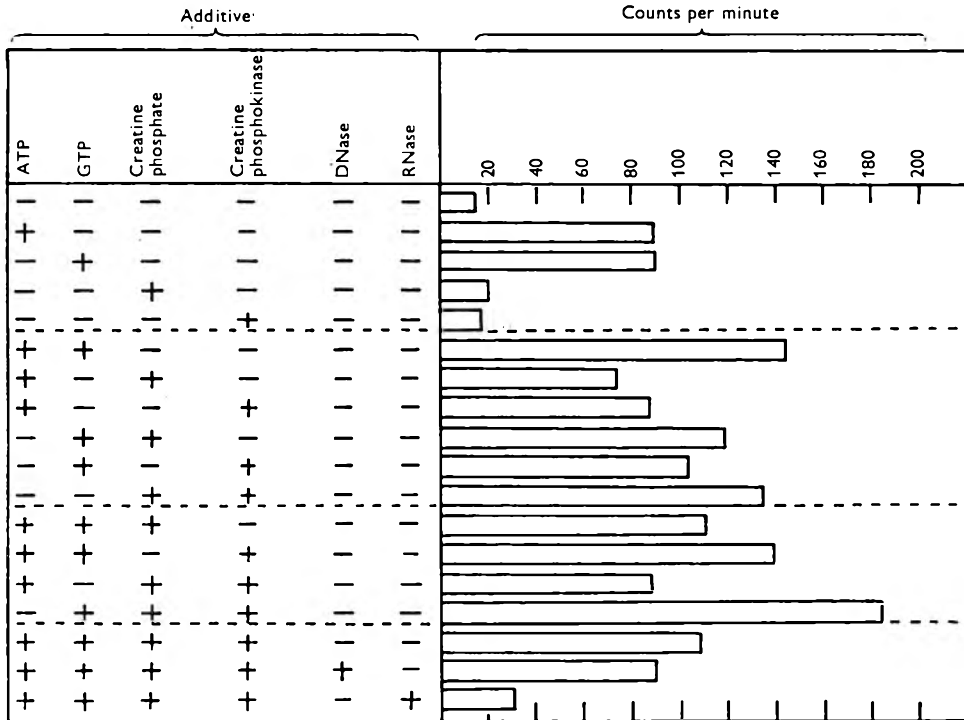

Fig. 1. The effect of various additives on incorporation of L-valine-1-^{14}C into paramecium 10,000*g* supernatant fluid fraction. Experimental conditions: volume, 1·6 ml. Label, L-valine-1-^{14}C, 0·25 μc (specific activity, 9·8 μc/μmole). ATP, 5 μmoles; GTP, 0·5 μmole; creatine phosphate, 30 μmoles; creatine phosphokinase, 100 μg. 10,000*g* supernatant fluid, 900 μg. protein. Sucrose, 250 μmoles; tris, 50 μmoles; KCl, 25 μmoles; $MgCl_2$, 5 μmoles. DNase, 10 μg.; RNase, 1 μg., pH 7·6. Incubated 55 min. at 33°. Incubation mixtures were then treated as described in Table 1.

Factors involved in the cessation of incorporation

The effect of repeated addition of 150,000*g* superantant fluid to the incubation system and the effect of pre-incubation of the system's components was examined. The data reported in Table 2 demonstrate that repeated addition of the supernatant fluid fraction caused no increased incorporation (samples 1–4). Pre-incubation of either the pellet fraction or the soluble fraction, in the presence and in the absence of co-factors, caused a marked decrease in incorporative power but did not destroy it. The same was true when the two fractions were incubated together before the introduction of co-factors (samples 5 to 7). The final set (no. 8) shows that significant loss of incorporative power occurred in the system when it was held at 0°.

Table 2. *Incorporation of L-valine-1-^{14}C into various incubation systems utilizing paramecium 150,000g pellet and supernatant fluid fraction**

Experimental conditions: volume, 1·4 ml. (final). Label, L-valine-1-^{14}C, 0·5 μc. (specific activity, 9·8 μc./μmole). GCC = GTP, 0·25 μmole + creatine phosphate, 10 μmoles + creatine phosphokinase, 100 μg. 150,000*g* pellet, 0·7 mg. protein; 150,000*g* supernatant fluid 3·5 mg. protein (final). Sucrose, 250 μmoles; tris, 50 μmoles; KCl, 25 μmoles; $MgCl_2$ 5 μmoles (all final). pH 7·6 Incubated at 33°. Following incubation 2 ml. cold medium A containing 5 mg. ^{12}C-DL-valine added followed by 4 ml. 10% (w/v) TCA.

	Time (min.)										
	0			10	20	30	40			80	
Sample no.	150,000*g* pellet (ml.)	150,000*g* supernatant fluid (ml.)	GCC	150,000*g* supernatant fluid (ml.)			150,000*g* pellet (ml.)	150,000*g* supernatant fluid (ml.)	GCC		Counts/min.
1a	0·5	0·5	+	–	–	–	Incubation stopped at 40 min.		.	.	957
1b	0·5	0·5	–	–	–	–			.	.	91
2a	0·5	0·4	+	0·1	–	–			.	.	1051
2b	0·5	0·4	–	0·1	–	–			.	.	79
3a	0·5	0·3	+	0·1	0·1	–			.	.	968
3b	0·5	0·3	–	0·1	0·1	–			.	.	82
4a	0·5	0·2	+	0·1	0·1	0·1			.	.	934
4b	0·5	0·2	+	0·1	0·1	0·1			.	.	80
5a	0·5	–	–	.	.	.	–	0·5	–	Incubation stopped at 80 min.	120
5b	0·5	–	–	.	.	.	–	0·5	+		440
5c	0·5	–	+	.	.	.	–	0·5	–		523
6a	–	0·5	–	.	.	.	0·5	–	–		83
6b	–	0·5	–	.	.	.	0·5	–	+		188
6c	–	0·5	+	.	.	.	0·5	–	–		237
7a	0·5	0·5	–	.	.	.	–	–	+		305
7b	0·5	0·5	–	.	.	.	–	–	–		113
8a†	–	–	–	.	.	.	0·5	0·5	+		753
8b†	–	–	–	.	.	.	0·5	0·5	–		56

* Repetitive additions of soluble fraction are shown in samples 1–4. Pre-incubation of components are shown in samples 5–8.
† Constituents held separately at 0° for 40 min. more than tubes 1a and 1b.

Distribution of ^{14}C-activity in the constituents of the 10,000g supernatant fluid

The distribution within the 10,000*g* supernatant fluid of incorporated ^{14}C-amino acid with respect to soluble and particulate components is given in Table 3. The total radioactivity of the untreated 150,000*g* pellet was 1170 d.p.m. (line 3 and line 4) *vs.* 260 d.p.m. (lines 7 and 8) in the 150,000*g* supernatant fluid [$(NH_4)_2SO_4$ precipitable, non-dialysable], i.e. less than 20 % of the activity appeared in the soluble protein. The picture was virtually identical in the material taken for pronase treatment—1330 d.p.m. (lines 5 and 6) *vs.* 338 d.p.m. (lines 9 and 10). Following the 37° incubation a considerable proportion of the activity in the 150,000*g* pellet and supernatant fluid not subject to pronase treatment was soluble in cold TCA. That this may have been due to endogeneous proteolytic activity is indicated by the fact that the sum of the activities of the 150,000*g* pellet and the soluble fraction ($\frac{1}{2}\Sigma$ lines 3–10) was 102 % that of the whole 10,000*g* supernatant fluid (TCA-insoluble and worked up for counting immediately after incorporation) and the fact that the label was present after dialysis. The addition of pronase to the fractions caused an 88 % decrease of activity in the TCA precipitate of the 150,000*g* pellet and an 80 % decrease in the radioactivity of TCA precipitate of the 150,000*g* supernatant fluid.

Table 3. *The effect of pronase on the distribution of ^{14}C activity in the 150,000g pellet and 150,000g supernatant fluid obtained from a paramecium 10,000g supernatant fluid fraction incubated with L-alanine-1-^{14}C*

Experimental conditions: nine 1·4 ml. incubation mixtures (7·0 mg. protein/tube) set up as described in Table 2 eight with GCC (GTP, 0·25 μmole + creatine phosphate, 10 μmoles + creatine phosphokinase, 100 μg.). pH 7·6, one without. Incubated 40 min. at 33°. Label, L-alanine-1-^{14}C, 0·5 μc. (specific activity, 7·5 μc./μmole). Following incubation two tubes (one +GCC, one −GCC) were brought to 5 % (w/v) TCA and then prepared for counting. The contents of the remaining seven tubes were pooled and handled as described in Methods. All counts are corrected, using internal standards, to 100 % efficiency.

Sample		D.p.m./10 mg. 10,000*g* supernatant protein
Whole 10,000*g* supernatant fluid	+GCC	1520
	−GCC	230
Untreated 150,000*g* pellet	TCA-precipitate	580
	TCA-supernatant fluid	590
Pronase-treated 150,000*g* pellet	TCA-precipitate	70
	TCA-supernatant fluid	1260
Untreated 150,000*g* supernatant fluid	TCA-precipitate	40
	TCA-supernatant fluid	220
Pronase-treated 150,000*g* supernatant fluid	TCA-precipitate	8
	TCA-supernatant fluid	330

Interaction of paramecium and mouse liver fractions

The data in Table 4 show that both the 150,000*g* supernatant fluid and the 'pH 5 fraction' from mouse liver were able to act in conjunction with paramecium 150,000*g* pellet to give amino acid incorporation. Also, the paramecium soluble fraction stimulated incorporation by twice-washed microsomes (the sum of the separate fractions was

40 C.P.M. *vs.* 149 C.P.M. for the combined system) but inhibited incorporation by unwashed mouse microsomes.

Action of inhibitors on amino acid incorporation

The effect of streptomycin, chloramphenicol and puromycin on amino acid incorporation by paramecium 10,000*g* supernatant fluid is given in Table 5. Chloramphenicol and streptomycin had little effect on incorporation, in agreement with previous findings with non-bacterial systems (Rendi & Ochoa, 1962; So & Davie, 1963; Marcus & Feeley, 1965), while puromycin down to a concentration of 0·001 μmole/ml. caused significant inhibition.

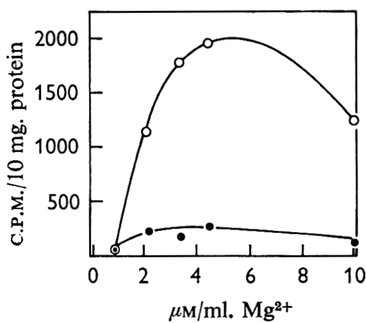

Fig. 2

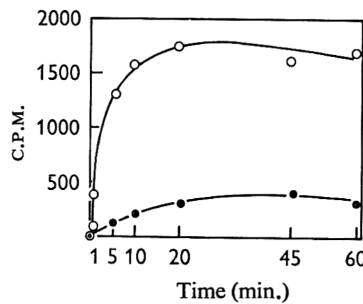

Fig. 3

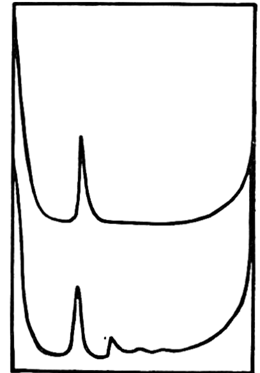

Fig. 4

Fig. 2. The effect of Mg^{2+} on L-leucine-1-^{14}C incorporation into paramecium 10,000*g* supernatant fluid fraction. With GCC, ○——○; Without GCC, ●——●. Experimental conditions: aliquots of packed cells were suspended in five medium A's differing in [Mg^{2+}]. From each suspension a 10,000*g* supernatant fluid was obtained and its [Mg^{2+}] determined. Incubation volume 1·4 ml. Label, L-leucine-1-^{14}C, 0·25 μc (specific activity, 7·1 μc/μmole). GCC = GTP, 0·25 μ mole+creatine phosphate, 10 μ moles+creatine phosphokinase, 100 μg. Sucrose, 250 μmoles; tris, 50 μmoles; KCl 25 μmoles, pH 7·6. Incubated at 33° for 30 min. Following incubation mixtures were treated as described in Table 1 except that ^{14}C-DL-leucine replaced valine.

Fig. 3. Time-course study of L-valine-1-^{14}C incorporation into paramecium 10,000*g* supernatant fluid fraction. With GCC, ○——○; Without GCC, ●——●. Experimental conditions: volume, 1·4 ml. Label L-valine-1-^{14}C, 0·5 μc. (specific activity, 9·8 μc./μmole). GCC = GTP, 0·25 μmole+creatine phosphate, 10 μmole+creatine phosphokinase, 100 μg. 10,000*g* supernatant fluid, 5·2 mg. protein. Sucrose, 250 μmoles; tris, 50 μmoles; KCl, 25 μmoles, $MgCl_2$, 5 μmoles, pH 7·6. Incubated 33°. Following incubation the mixtures were treated as described in Table 1.

Fig. 4. Analytical ultracentrifugation of paramecium 10,000*g* supernatant fluid fraction. Speed, 31,410 rev./min.; diaphragm angle, 55°; temperature, 2·7°; solvent, medium A. Top: 1·0 μg./ml. RNase added. Bottom: control. Photo taken 21 min. after reaching speed and 57 min. after adding enzyme. Sedimentation is from left to right. Major peak = 80s.

Demonstration of polyribosomes in paramecium

Routine examination in the analytical ultracentrifuge of paramecium 10,000*g* supernatant fluids showed the presence of a relatively large 80s boundary. In addition, smaller boundaries representing ribosomal dimers, trimers and tetramers could be distinguished and occasionally the pentamers were resolved. To determine whether or

not the aggregates were polyribosomes, a portion of a 10,000*g* supernatant fluid was mixed with RNase (concentrations of the enzyme were 0·5 and 1·0 μg./ml.) and the mixtures held below 2·7° throughout the experiment. Figure 4 shows that the RNase treatment eliminated the faster-sedimenting boundaries and that the area beneath the 80s boundary increased. The only difference noted between the 0·5 μg./ml. RNase

Table 4. *Interaction of paramecium and mouse liver components in incorporation of L-leucine-1-^{14}C*

Experimental conditions: volume, 1·5 ml. Label, L-leucine-1-^{14}C, 0·25 μc. (specific activity, 10 μc./μmole). AGCC = ATP, 2·5 μmoles + GTP, 0·25 μmole + creatine phosphate, 10 μmoles + creatine phosphokinase, 100 μg. Protein = paramecium 150,000*g* pellet, 1·2 mg.; paramecium 150,000*g* supernatant fluid, 2·5 mg.; mouse unwashed 150,000*g* pellet, 4·1 mg.; mouse twice washed 150,000*g* pellet, 2·5 mg.; mouse 150,000*g* supernatant fluid, 26·5 mg.; mouse 'pH 5 fraction', 0·7 mg. Sucrose, 250 μmoles; tris, 50 μmoles; KCl, 25 μmoles; $MgCl_2$, 5 μmoles. pH 7·6. Incubated 45 min. at 33°. Incubation stopped with the addition of 2 ml. medium A containing 5 mg. ^{12}C-DL-leucine followed by 5 ml. 7% (w/v) TCA.

Paramecium 150,000*g* pellet	Paramecium 150,000*g* supernatant fluid	Mouse-liver 150,000*g* pellet	Mouse-liver 150,000*g* supernatant fluid	Mouse-liver 'pH 5' fraction	Counts per min.	
					+AGCC	−AGCC
+	−	−	−	−	29	12
−	+	−	−	−	20	18
−	−	(Unwashed)	−	−	650	34
−	−	(Twice washed)	−	−	20	17
−	−	−	+	−	25	15
−	−	−	−	+	49	36
+	+	−	−	−	111	19
+	−	−	+	−	137	21
+	−	−	−	+	444	31
−	+	(Unwashed)	−	−	213	32
−	+	(Twice washed)	−	−	149	23
−	−	(Unwashed)	+	−	1245	23
−	−	(Unwashed)	−	+	1702	35

Table 5. *The effect of inhibitors on incorporation of L-alanine-1-^{14}C by paramecium 10,000g supernatant fluid fraction*

Experimental conditions: volume, 1·5 ml. Label, L-alanine-1-^{14}C, 0·25 μc. (specific activity 12·3 μc./μmole). GCC = GTP, 0·25 μmole + creatine phosphate, 10 μmoles + creatine phosphokinase, 100 μg. 10,000*g* supernatant fluid, 8·3 mg. protein. Sucrose, 250 μmoles; tris, 50 μmoles; KCl, 25 μmoles; $MgCl_2$, 5 μmoles pH 7·6. Incubated 35 min. at 32°; reaction stopped by adding of 1·5 ml. 10% (w/v) TCA.

Inhibitor (μmole/ml.)	Counts per min.	% inhibition
None	388	0
−GCC	70	82
Streptomycin 0·1	322	17
Streptomycin 0·01	404	−4
Chloramphenicol 0·1	353	9
Chloramphenicol 0·01	332	14
Puromycin 0·1	97	75
Puromycin 0·01	203	48
Puromycin 0·001	243	37

treatment and the 1·0 μg./ml. treatment was that in the former a small amount of dimer was still present in the early stages of the run.

Incorporation of amino acid into gradient fractions of paramecium 10,000g superantant fluid

To determine whether ribosomal activity could be directly shown in paramecia, the experiments described in Fig. 5 were made. The data from the 60 min. centrifugation (Fig. 5*a*) indicate a measure of activity in the lower fractions of the gradient. Similar

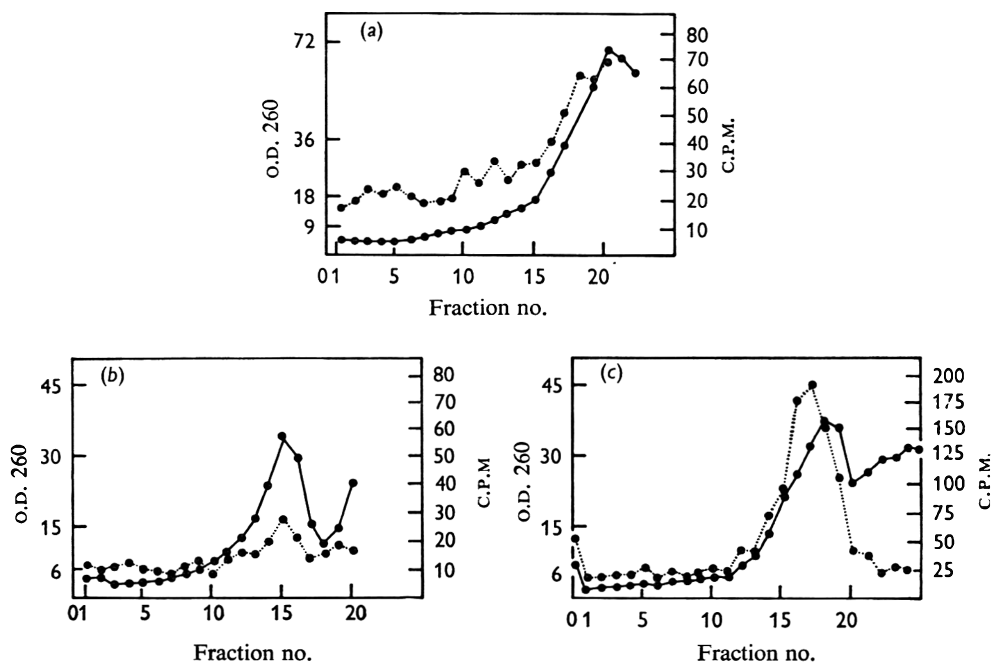

Fig. 5. Distribution of incorporation activity in sucrose density gradient centrifugations of paramecium 10,000*g* supernatant fluid fraction. Counts per minutes (C.P.M.), ●····●; optical density (O.D.) at 260 mμ, ●—●. Experimental conditions: 15–30% linear sucrose gradients made up in medium A were overlayered with paramecium 10,000*g* supernatant fluid and centrifuged in the Spinco SW 25-1 rotor at 53,000*g* for 60 min. (5*a*), 120 min. (5*b*) or 90 min. (5*c*). 1 ml. samples were removed through the bottom of the tubes for incubation. Incubation: volume, 1·5 ml. Label, (*a*, *b*) L-leucine-1-^{14}C, 0·25 μc. (specific activity 10 μc./mole); (*c*) L-alanine-1-^{14}C, 0·5 μc. (specific activity, 7·5 μc./mole). ATP, 2·5 μmoles; GTP, 0·25 μmole; creatine phosphate, 10 μmoles; creatine phosphokinase, 100 μg. tris, 50 μmoles; KCl, 25 μmoles; $MgCl_2$, 10 μmoles; Sucrose 490 μ to 75 μmoles. Paramecium 150,000*g* supernatant fluid, 1·1 mg. protein (*a*, *b*). Mouse liver 'pH 5 fraction', 0·27 mg. protein (*c*), pH 7·6. Incubated 45 min. at 33°. Following incubation 2 ml. cold medium A added containing 5 mg. bovine γ globulin and 5 mg. ^{12}C-DL-leucine (*a*, *b*) or ^{12}C-DL-alanine (*c*) followed by 5 ml. 7% (w/v) TCA.

observations were made on a 30 min. centrifugation. In the 120 min. centrifugation (Fig. 5*b*) the 80s ribosomes had moved well away from the top of the gradient and there was a correlation of amino acid incorporation with them. The use of mouse liver 'pH 5 fraction' also caused incorporation which appeared with the 80s peak (Fig. 5*c*).

The effect of Mg^{2+} on the distribution of ^{14}C-amino acid associated with the 80s ribosomes

Amino-acid-labelled 80s ribosomes from paramecia were prepared as described under Methods. The results appear in Fig. 6. Comparable material was also examined in an analytical ultracentrifuge. Figure 6*a* (10^{-2}M-Mg^{2+}) shows excellent correlation

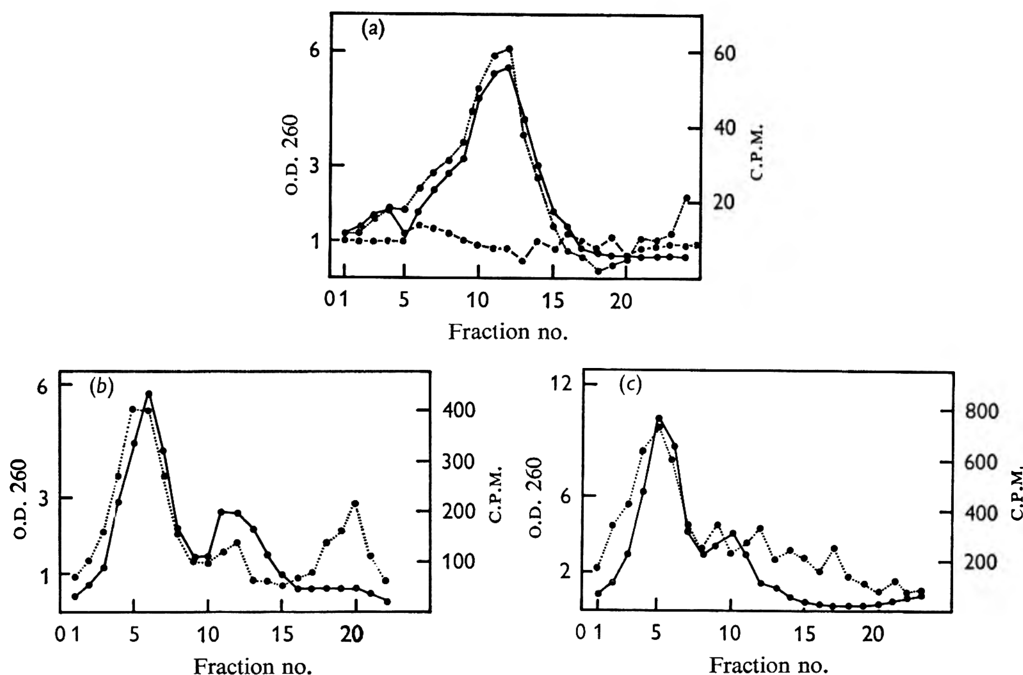

Fig. 6. Effect of [Mg^{2+}] and dialysis time on the distribution of 80s ribosomal material and associated amino acid in sucrose density gradients. C.P.M., ●·····●; C.P.M. (without AGCC), ●—●. ●—●; O.D. 260 mμ,

Experimental conditions:

(*a*) Incubation-volume, 27 ml. Label, 9 μc. L-alanine-1-^{14}C (specific activity 7·5 μc./μmole). AGCC = ATP, 45 μmoles + GTP, 4·5 μmoles + creatine phosphate, 180 μmoles + creatine phosphokinase, 1·8 mg. Paramecium 150,000*g* pellet, 30 mg. protein; mouse liver 'pH 5 fraction', 26 mg. protein. Tris, 900 μmoles; KCl, 450 μmoles; $MgCl_2$, 90 μmoles pH 7·6. Incubated 45 min. at 33°. After obtaining purified 80s ribosomes as described in Methods, they were dialyzed 15 hr against two changes of medium A, $Mg^{2+} = 10^{-2}$ M, no sucrose. The impermeate was layered on a 5–20% linear sucrose gradient in medium A $Mg^{2+} = 10^{-2}$ M and centrifuged two hr at 53,000 *g* in the Spinco SW 25–1 rotor.

(*b*) Incubation-volume, 8·4 ml. Label, 3 μc. reconstituted protein hydrolysate. GTP, 1·5 μmoles; creatine phosphate, 60 μmoles; creatine phosphokinase 0·6 mg. Paramecium 10,000*g* supernatant fluid, 35 mg. protein. Sucrose, 1·5 μmoles; tris, 300 μmoles; KCl, 150 μmoles; $MgCl_2$, 30 μmoles, pH 7·6. Incubated 30 min. at 33°. After obtaining purified 80s ribosomes as described in Methods, they were dialysed 15 hr against two changes of medium A containing no sucrose and no Mg^{2+}. The impermeate was layered on a 5 to 20% sucrose gradient in medium A (no Mg^{2+}) and centrifuged seven hr at 53,000*g*.

(*c*) Incubation-volume, 7 ml. Label 2·5 μc. reconstituted protein hydrolyzate. GTP, 1·25 μmoles; creatine phosphate, 50 μmoles; creatine phosphokinase, 0·5 mg. Paramecium 10,000*g* supernatant fluid, 50 mg. protein. Sucrose, 1·25 mmoles; tris, 250 μmoles; KCl 125 μmoles; $MgCl_2$, 25 μmoles, pH 7·6. Incubated 35 min. at 31°. Following incubation the preparation was handled as 6*b* except that it was dialysed 4·5 hr against three changes of buffer before layering on the 5–20% gradient.

between the absorbency and radioactivity curves. The analytical ultracentrifuge showed that the majority of material was present in the 80s form but that a considerable degree of spontaneous polymerization had taken place after overnight dialysis against 10^{-2} M-Mg^{2+}. This was substantiated by the marked shoulder seen between fractions 5 and 9 of Fig. 6*a*. Figure 6*b* shows the distribution of material from 80s ribosomes subjected to dialysis for 15 hr against buffer deficient in Mg^{2+} followed by centrifugation for 7 hr, while Fig. 6*c* shows the distribution obtained when dialysis was for 4·5 hr. The analytical ultracentrifuge showed the presence of 45s and 30s boundaries. While over 95% of the TCA-insoluble label present in the initial 80s material was recovered from the gradients shown in Fig. 6*a* and 6*c*, only two-thirds of that present initially in the experiment shown in Fig. 6*b* was recovered.

DISCUSSION

The experiments described indicate that *Paramecium aurelia* stock 51 was able to incorporate amino acid into protein via the ribosomal route. For incorporation to occur the 150,000*g* supernatant fluid (soluble fraction) was required together with guanosine triphosphate (GTP) and either adenosine triphosphate (ATP) or an ATP-generating system (Table 1; Fig. 1). Furthermore, the stimulation by GTP and ATP (or the ATP generating system) supplemented one another (Fig. 1), suggesting that these compounds played different roles in incorporation as would be expected from the presently accepted model of protein biosynthesis. While the paramecium system was essentially unaffected by DNase treatment, it showed a marked sensitivity to submicrogram amounts of RNase, indicating a requirement for labile RNA, e.g. messenger RNA. That the protease pronase (Nomoto, Narahashi & Murakami, 1960) released the label into the cold TCA supernatant fluid and that puromycin inhibited incorporation into the TCA precipitate support the conclusion that the amino acid was incorporated into protein via peptidyl-RNA intermediates (Allen & Zamecnik, 1962; Zamecnik, 1962).

The time-course of incorporation in Fig. 3 shows a rapid decay in the rate of incorporation similar to that reported by Chesters (1966) for *Crithidia oncopelti*. That this decay was not alleviated by repeated additions of soluble fraction to the system is shown in Table 2 (samples 1–4). It appears unlikely, therefore, that the paramecium system was capable of much *de novo* protein synthesis. More probably polypeptides present when incubation began were augmented, and any chain initiation which occurred was not carried to completion of the polypeptide. The relatively small amount of label (< 20%) found in the protein of the soluble fraction after incubation (Table 3, 150,000*g* supernatant fluid) is additional support for this conclusion.

That contaminating micro-organisms played no significant part in incorporation is indicated by the following observations: the mass cultures used for these studies were free from bacterial and fungal contaminants; soluble fraction, 150,000*g* pellet and cofactors were all required for incorporation; incorporation ceased after incubation for 10 min.; the ^{14}C-label became associated with 80s ribosomes.

The experiments reported in Table 2 (samples 5–8) show that pre-incubation of the soluble fraction together with the ribosomal fraction caused a two-thirds loss in activity (305 *vs*. 957 C.P.M.) while a somewhat less severe loss occurred when ribosomes alone were pre-incubated. These findings are substantially in agreement with those of

Marcus & Feeley (1965) who worked with a cell-free system from peanut cotyledon and are characteristic of ribosomally-controlled amino acid incorporation systems (Allen & Schweet, 1962; Matthaei & Nirenberg, 1961). However, pre-incubation of paramecium soluble fraction alone caused a severe decrease of activity in contrast to Marcus & Feeley's results. When this observation is considered in conjunction with the finding that paramecium soluble fraction caused marked inhibition of amino acid incorporation by unwashed mouse liver microsomes, while mouse liver 'pH 5 fraction' allowed a fourfold increase in incorporation by paramecium ribosomes (as compared with paramecium soluble fraction, Table 5), we have a clear indication of the presence of inhibitors in the paramecium 150,000*g* supernatant fluid. Furthermore, it appears that during incubation destruction of essential components occurred.

The finding that mouse liver 150,000*g* supernatant fluid and 'pH 5 fraction' (Table 4) stimulated amino acid incorporation by paramecium ribosomes and that paramecium soluble fraction stimulated incorporation by washed mouse microsomes, is a direct indication that the paramecium system follows the classical model. However, not until specific proteins are isolated from these hybrid systems will it be possible to determine whether mouse messenger RNA combines with free paramecium ribosomes to make mouse protein, or whether mouse transfer RNA supplies amino acids to paramecium messenger RNA.

Evidence which implicates the paramecium ribosomes in incorporation comes from the sucrose density gradient experiments (Figs. 5, 6). Figure 5 (*b*, *c*) shows a correlation of radioactivity with the 80s material, while Fig. 5*a* indicates that some active polyribosomes might have been present in the gradient. The substitution of mouse 'pH 5 fraction' (Fig. 5*c*) for paramecium soluble fraction caused a distinct shift of the radioactivity peak (relative to 260 mμ extinction) toward the lower end of the gradient. This shift may have been due to the presence of inhibitors from the paramecium soluble fraction which contaminated the higher fractions of the gradient, for no such shift is seen in Fig. 6*a* which utilized paramecium 150,000*g* pellet and mouse 'pH 5 fraction'. Figure 6*a* shows that a correlation between radioactivity and 260 mμ extinction persisted when purified amino acid-labelled 80s ribosomes formed spontaneous polymers at 10^{-2} M-Mg^{2+}. Upon dissociation of the 80s particles into 45s and 30s subunits (Fig. 6*b*, *c*) little label was associated with the smaller subunit, while the 45s unit retained marked radioactivity. While the specific activity of the 45s unit was not appreciably affected by prolonged dialysis (Fig. 6*b* *vs.* 6*c*), one-third of TCA precipitable label present in the initial material was lost; and the released protein is seen to peak in fraction 20. In the gradient of the 4·5 hr dialysis essentially all of the TCA precipitable label present initially was recovered and in place of a peak near the top of the gradient there is a trail of radioactivity through the top half of gradient. These observations suggest that a considerable fraction of the nascent protein was tightly bound to the 45s unit and was neither degraded nor released by prolonged exposure to low Mg^{2+} concentrations. However, the nascent protein which was released by low Mg^{2+} concentrations reflects prolonged exposure in a change of position in the gradient and by a significant loss of material. Possibly these findings indicate that in the experiment shown in Fig. 6*c* the nascent protein was released from one or the other of the subunits only during centrifugation. If proteolytic activity during extended dialysis was responsible for the loss of label, release of the nascent protein only during the course of centrifugation might have effectively protected against such

activity. Alternatively there might have been a degree of disintegration associated with ribosomal dissociation. Initially the released protein might have been bound to ribosomal fragments from which it later dissociated. Peptides too small to have been retained during dialysis or precipitated by TCA would have been lost.

Tashiro & Siekevitz (1965), who used guinea-pig hepatic ribosomes pulse-labelled *in vivo* with amino acid and dissociated with a chelator, reported results virtually identical with the pattern shown in Fig. 6*b*, while Philipps (1966) with rabbit reticulocyte ribosomes presented evidence indicating that upon ribosomal dissociation the nascent protein might be bound to either subunit. In this connexion it is interesting to note that the reticulocyte ribosomes dissociated to give compact 60s and 40s subunits, while paramecium ribosomes dissociate into the more open 45s and 30s forms (A. H. Reisner & H. Macindoe, unpublished results). In the latter system the secondary bonds which tend to bind the polypeptide to both of the subunits may become disrupted allowing the peptidyl-RNA-ribosomal bond to play a more dominant role.

Concerning the sensitivity of the protein synthetic mechanism in paramecium to [Mg^{2+}] (Fig. 2) a similar optimum and narrowness of range is noted by Chesters (1966) in the only other protozoan examined. It is noteworthy that at concentrations below 3 μmoles/ml. Mg^{2+}, where incorporation shows a very sharp decline, examination of ribosomal structure in paramecium (A. H. Reisner & H. Macindoe, unpublished results) reveals that the 80s ribosomes ($S_{o_{20w}}$ = 84·5s) undergo marked hydration. Thus at 1 μmole/ml. Mg^{2+} about 75% of the ribosomes are in a hydrated 60s form (no 45s or 30s material is detectable). Polyribosomes are still present at this Mg^{2+} concentration but have reduced sedimentation coefficients. The reduction of amino acid incorporation into protein within the range of 1 to 4 μmoles/ml. Mg^{2+} may be, therefore, a direct consequence of ribosomal disorganization.

We are indebted to Dr W. J. van Wagtendonk for a single-membered culture of paramecia, for testing our early baker's yeast preparations and for suggestions in developing the new culture medium. Our thanks are due to Dr J. K. Chesters for permission to refer to his manuscript prior to publication and to Misses R. Price and D. Bramfitt for able technical assistance.

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Taxonomy of Anaerobic Thiobacilli

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SUMMARY

The taxonomic position of *Thiobacillus denitrificans* was investigated by using six newly isolated strains which were compared with other species of this genus, under aerobic and anaerobic growth conditions. The validity of *T. denitrificans* is shown. *Thiobacillus intermedius* and *T. thermophilica*, two species recently described by other authors, were also investigated.

INTRODUCTION

The species *Thiobacillus denitrificans* was first described by Beijerinck (1904*a*, *b*) and said to be capable of autotrophic growth; aerobically in the presence of thiosulphate or thiocyanate and anaerobically in the presence of thiosulphate and nitrate. In previous studies of the genus *Thiobacillus* (Hutchinson, Johnstone & White, 1965, 1966) many strains were isolated from a wide variety of sources, but strains corresponding to *T. denitrificans* were not found. As aerobic enrichment cultures had been used in these experiments the present paper describes attempts to isolate *T. denitrificans* using anaerobic enrichment cultures and isolation techniques. Twenty sources were examined and strains which produced gas from nitrate-containing media isolated in pure culture. These organisms proved to be facultative anaerobes but unlike the strains described by Baalsrud & Baalsrud (1954) and Woolley, Jones & Happold (1962) they did not lose their ability to grow anaerobically after cultivation for a period of 6 months. During this period the strains were subcultured at 21-day intervals.

As with the acidophilic species previously studied (Hutchinson *et al.* 1966) there was considerable difficulty in deciding the test conditions to be used when comparing these strains with the other species. There appeared to be no logical method for selecting either aerobic or anaerobic test conditions and therefore these organisms were compared with closely related species in two separate series of tests under aerobic and anaerobic conditions.

During the course of this work two rather unusual species of the thiobacilli were described by other authors. *Thiobacillus intermedius* (London, 1963) was an acidophilic facultative heterotroph which decreased the pH value of thiosulphate media to below pH 2·8; a culture of the original strain together with a similar organism isolated by us have been included in the aerobic series of tests. The other species, *T. thermophilica* (Egorova & Deryugina, 1963), is a spore-forming autotrophic thermophil; our examination of this organism was limited to confirming the original description.

METHODS

Organisms. As no authentic strains of *Thiobacillus denitrificans* were available to us and no isolates corresponding to the original description had been found by us in previous work (Hutchinson *et al.* 1965, 1966), twenty sources were examined specifically for anaerobic thiobacilli. Enrichment cultures of the various samples were set up in the S 6 thiosulphate medium (Hutchinson *et al.* 1965) with the addition of $NaHCO_3$ 0·5 g./l. as carbon source and KNO_3 5·0 g./l. as oxidant (medium S 8). These cultures were contained in completely filled and stoppered bottles.. When gas formation was observed in the cultures two-thirds of the culture medium was replaced aseptically with fresh medium. This accelerated the rate at which the gas accumulated and was

Table 1. *Sources which yielded nitrogen-forming enrichment cultures on anaerobic incubation*

Source	pH value of source	Isolates
Lagoon system, carbonization effluent	—	8G, 9G, 10G, 11G
Lagoon system, domestic sewage	7·0	1s, 2s, 3s, 4s
Anaerobic digestion tank (1), domestic sewage	6·8	2T, 2T′, 3T, 4T
Anaerobic digestion tank (2), domestic sewage	6·4	1U, 2U
Biological filter, carbonization effluent	7·1	1W*
Fertile soil	6·7	—†
Activated sludge plant, carbonization effluent	7·1	—†

* Could not be obtained in pure culture.
† No nitrogen-forming strains could be isolated.

Table 2. *Aerobic strains*

Collection or source	Species	Code number
NCIB 8370	*Thiobacillus thioparus*	b5
P. A. Trudinger	*T. neapolitanus*	c1
F. C. Happold	*T. thioparus*	h1
		h2
		h3
D. P. Kelly	*T. neapolitanus*	k1
J. London	*T. intermedius*	l1
Mrs M. Townshend	*T. neapolitanus*	m1
Miss E. S. Pankhurst	*T. thioparus*	p1
T. G. Tomlinson	*T. thioparus*	t1
W. Vishniac	*T. neapolitanus*	v1
New isolate	*T. thioparus*	1B
New isolate	*T. intermedius*	2R

accompanied by the deposition of elementary sulphur. At this stage the cultures were plated on S 8 medium agar and incubated at 28° in a McIntosh & Fildes jar. All the different types of colonies seen were purified by three consecutive single-colony isolations, and all the isolates except 2T′ were maintained under anaerobic conditions before testing. Gas formation was observed in only six of the twenty enrichment cultures examined. As may be seen from Table 1, successful isolations (14) were limited to four sources. De Kruyff, van der Walt & Schwartz (1957) and Woolley *et al.* (1962) reported similarities between *Thiobacillus denitrificans* and *T. thioparus*; therefore representative strains of the latter species were included in the tests. The authentic

strain of *T. intermedius*, very kindly supplied by Dr J. London, and a very similar strain isolated in our laboratoties were also included in the aerobic series of tests. These organisms and their origins are listed in Table 2.

Tests. All isolates were examined under aerobic and anerobic conditions according to the standard test scheme used previously. The anaerobic tests were made in the anaerobic medium (S 8) in completely filled and stoppered bottles.

Some of the tests did not differentiate between the organisms under study and were therefore omitted from the analysis. Of the 30 tests investigated anaerobically only 18 were finally utilized and these yielded 44 character states. In the aerobic series only 24 out of 40 tests were used giving 62 character states.

All measurements and analyses were made as in previous studies (Hutchinson *et al.* 1965, 1966). The two sets of data were analysed independently though certain tests were common to both series of scorings. The tests used are summarized in Table 3.

Table 3. *Tests*

Test compound or variable	Concentration (%)	Comments	Characters Aerobic	Characters Anaerobic
Sulphur (excess)		*	3	2
Ammonium thiocyanate	0·02	*	2	2
Hydrogen sulphide		*	2	—
Thioacetamide	0·02	*	3	—
Nutrient medium		*	3	—
19°		Rate of thiosulphate oxidation	2	—
29°		*	2	—
35°		*	2	—
29°		Amount of thiosulphate oxidation	4	2
pH 8·25		Amount of thiosulphate oxidation	—	2
S6 or S8 medium agar		Sulphur deposition	2	3
S8 medium agar		Growth in McIntosh & Fildes jar	2	—
S8 medium		Gas production	—	2
		Formation of nitrite	—	3
		Deposition of sulphur	—	3
S6 medium		Final pH value	4	—
Bacitracin		*	2	—
Novobiocin		*	2	2
Streptomycin		*	—	2
Chloramphenicol		*	—	2
Mixed phosphate	4	*	3	—
Sodium chloride	5	*	2	—
Potassium nitrate	1	*	3	3
Ammonium chloride	2·5	*	2	3
Ammonium thiocyanate	0·02	*	2	2
Phenol	0·01	*	—	3
	0·02	*	4	3
Sodium glutamate	1	*	3	2
Nutrient broth	1	*	3	3
Sulphur	Excess	*	3	—
Calcium chloride	1	*	2	—

* Previously described (Hutchinson *et al.* 1965).

Chemical analysis. The amount of thiosulphate utilized was determined by titration with 0·01 N-iodine, and gas formation (presumed to be nitrogen) by visual observation.

Analysis of data. The results were scored according to the method of Beers & Lockhart (1962) and the S values calculated by the method of Sneath (1957). The order of the rearranged matrices was obtained by inspection.

RESULTS

The results of the numerical analyses for the aerobic and anaerobic test series are shown in Figs. 1 and 2, in which the strains have been arranged in order of similarity. The strains may be segregated into four groups reported previously (Hutchinson *et al.* 1965): 0 (the trautwein types), 3 (*Thiobacillus thioparus*) and 4 (*T. neapolitanus*) together with a newly formed group containing the anaerobic denitrifying thiobacilli which has been termed group 2.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	
1	1U	00	—	—	—	—	—													group 2
2	2T	89	00	—	—	—	—													
3	9G	89	00	00	—	—	—													
4	8G	72	83	83	00	—	—													
5	2T	71	71	71	82	00	—													
6	2S	71	65	65	82	87	00													
7	1B	39	39	39	28	18	12	00	—	—	—	—	—	—	—	—				group 3
8	3T	44	44	44	39	29	24	83	00	—	—	—	—	—	—	—				
9	p1	28	39	39	44	24	35	67	72	00	—	—	—	—	—	—				
10	h1	33	28	28	44	29	35	72	72	78	00	—	—	—	—	—				
11	b5	20	13	13	27	27	29	67	60	80	87	00	—	—	—	—				
12	1S	22	17	17	17	12	18	78	72	67	72	87	00	—	—	—				
13	4T	28	22	22	28	29	29	72	78	67	72	80	83	00	—	—				
14	h3	17	8	8	8	9	9	67	58	50	58	75	83	92	00	—				
15	11G	33	28	28	28	24	24	56	50	39	50	53	72	56	50	00	—	—	—	group 0
16	4S	28	28	28	28	18	18	56	50	39	44	33	44	39	42	67	00	—	—	
17	m1	9	9	9	9	10	9	45	36	36	36	37	55	36	40	82	91	00	—	group 4
18	c1	12	12	12	12	14	12	50	37	37	37	37	50	37	40	87	00	00	00	

Fig. 1. Re-arranged matrix of S values–anaerobic organisms tested anaerobically.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	
1	4S	00	—	—	—												group 0
2	11G	54	00	—	—												
3	10G	54	96	00	—												
4	2S	50	50	50	00	—	—										group 2
5	8G	46	50	46	92	00	—										
6	9G	42	46	42	83	92	00										
7	2T	42	46	42	83	92	00	00									
8	h2	42	50	46	67	71	79	79	00	—	—	—					group 3
9	t1	37	50	46	67	75	75	75	87	00	—	—					
10	h1	33	37	33	54	62	71	71	83	87	00	—					
11	b5	33	37	33	54	58	67	67	87	83	92	00					
12	2R	42	50	46	54	54	62	62	71	62	62	67	00	—	—		group 7
13	l1	37	62	58	50	50	50	50	62	62	58	54	71	00			
14	v1	21	25	21	21	25	33	33	50	37	42	46	42	46	00	—	group 4
15	k1	21	25	21	21	25	33	33	50	37	42	46	42	46	00	00	

Fig. 2. Re-arranged matrix of S values–anaerobic organisms tested aerobically.

Although similarities exist between group 2 and group 3 especially in their ability to oxidize thiocyanate, the differentiation between these two groups is shown in Table 4. These data indicate that there is no overlap between the highest observed S values for one group and the lowest S value for the other. Moreover, with the exception of case 4 in Table 4, these two groups are distinct even if one adopts the criteria of a probable distribution of S values, plus or minus three standard deviations about the mean for the median organism of either of the two groups. This differentiation was also supported by certain cultural characteristics. These were:

(1) Only members of group 2 were able to oxidize thiocyanate anaerobically.

(2) Only members of group 2 were capable of active denitrification in a nitrate-containing liquid medium under anaerobic conditions. Although certain other strains, notably group 3 and group 0, grew under these conditions to a limited extent, they did not possess the ability to denitrify actively producing visible amounts of nitrogen gas.

(3) Members of group 2 differed from those of group 3 in that the final pH values attained during aerobic cultivation were never below pH 5·0 with sulphur or thiosulphate as substrate. When the substrate was thiosulphate the amount oxidized was limited by the acidity resulting from the oxidation. For example in medium S 6, 18% of the thiosulphate was removed by these organisms in 8 days when the pH value had fallen to 5·45 and this was unchanged after incubation for a further 20 days. Similarly, cultures inoculated into media containing 0·5, 1, 2% (w/v) thiosulphate, oxidized in 28 days 31%, 15% and 6%, of the initial thiosulphate, the corresponding final pH values were 5·35, 5·4 and 5·6. All members of group 3 under aerobic conditions oxidized all the thiosulphate in medium S 6 with a final pH value between 3·5 and 4·0.

Table 4. *Highest and lowest S values to the centrotypes of group 2 and group 3*

	Group 2			Group 3		
	High	Mean	Low	High	Mean	Low
A. Anaerobic tests						
(i) 8G (centrotype of group 2)	83	80	72	44	29	9
(ii) 1s (centrotype of group 3)	22	17	12	87	77	67
B. Aerobic tests						
(iii) 8G (centrotype of group 2)	100	92	83	75	66	58
(iv) h2 (centrotype of group 3)	79	74	67	87	86	83

Table 5. *The sensitivity of groups 2 and 3 to certain antibiotics when tested under aerobic and anerobic conditions*

	Novobiocin		Streptomycin		Bacitracin	
	a	b	a	b	a	b
Group 2	R	R	S	R	S	S
Group 3	S	R	S	S	S	R

a, aerobic conditions; b, anaerobic conditions. R = resistant; S = sensitive.

(4) Differences exist between these two groups with respect to their antibiotic sensitivity patterns when examined by using Sentest sensitivity discs (Evans Medical

Ltd., Speke, Liverpool) on plate cultures incubated aerobically or anaerobically. The sensitivity patterns of both groups are given in Table 5. Moreover, the sensitivity pattern for these strains differed according to the conditions in a manner comparable to the findings of Kogut, Lightbown & Isaacson (1965) who found that *Escherichia coli* under anaerobic conditions was not inhibited by streptomycin because these conditions interfered with the uptake of this compound. A similar phenomenon may be operative here for group 3 organisms with respect to bacitracin and novobiocin.

The examination of *Thiobacillus intermedius* confirmed that this organism in addition to producing an acidity pH of 2·8 in thiosulphate or sulphur media also grew heterotrophically in common organic media. The isolate 2R was 71 % similar to the authentic culture and should probably be regarded as another strain of the same species.

Thiobacillus thermophilica, very kindly supplied by Dr A. A. Egorova, was not tested with the other strains because of its abnormal temperature requirement; growth did not occur at 37°, but at 57° small colonies were visible after 18 hr which increased to 5 mm. diam. in 10 days. Similar results were obtained in liquid culture and growth only occurred in the presence of either thiosulphate or sulphide. The amount of thiosulphate oxidized was small, only 8 % of a 0·5 % (w/v) thiosulphate medium, in 28 days. This was accompanied, however, by a slight fall in the pH value of the medium and a positive test for sulphate, indicating that some of the thiosulphate had been completely oxidized.

No growth could be obtained in any of the common organic media nor in the basal mineral salts medium without thiosulphate.

The vegetative cells were Gram-negative and rather larger than the other thiobacilli, 2–3 μ by 0·6–0·8 μ. Many terminal spores were observed and the chain formation as described by Egorova & Deryugina (1963) was very characteristic.

DISCUSSION

As may be seen in Fig. 1, the denitrifying group 2 appears distinct from the established species, *Thiobacillus thioparus* (group 3). There is considerable intragroup variation in the similarity values of these two groups which may be the result of the comparatively small selection of tests used in this instance. This is supported to some extent by the results for 2T and 2T′ which only resemble each other at 71 % level; these are the same strain but differed in that 2T′ was grown aerobically prior to testing.

Baalsrud & Baalsrud (1954) and Woolley *et al.* (1962) have expressed the view that with prolonged aerobic cultivation, *Thiobacillus denitrificans* reverts to a *T. thioparus* type. However, the instance of 2T′ quoted above would suggest that this organism resembles more closely the members of group 2 than those of group 3. Further evidence for this differentiation is shown in the physiological characters of denitrification in anaerobic culture, the ability to oxidize thiocyanate anaerobically and in aerobic culture a final reaction not below pH 5·0. The differentiation of groups 2 and 3 under aerobic conditions of cultivation (Fig. 2) is also evident. These data would strongly suggest that *T. denitrificans* is a valid species and distinct from the other named thiobacilli.

From the account of London (1963) and our experimental data there is little doubt that *Thiobacillus intermedius* is a member of the genus *Thiobacillus* and is probably sufficiently different from *T. thio-oxidans* to warrant species rank.

The problem of the classification of *T. thermophilica* is rather more complex for, although it appears to be an obligate autotroph which utilizes reduced sulphur compounds, the morphology of this organism is very different from that of the other thiobacilli. Although it cannot by definition be a member of the Pseudomonadales we suggest that it should be retained within the genus *Thiobacillus* until more evidence of its relationship is forthcoming. To some extent this situation is similar to that of the anaerobic sulphur reducing genus *Desulfovibrio* which included both monotrichous non-sporulating and peritrichous sporulating bacteria. In this group the sporulating organisms have been separated from the non-sporulating types and placed in a different genus (Campbell & Postgate, 1965). However, no indication was given of whether the two genera were to be separated by the removal of the new genus *Desulfotomaculum* from the Pseudomonadales.

A diagnostic table for the identification of all the species of the thiobacilli will be given in a later paper.

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A Quantitative Study of the Bacteria of a Temporary Pond

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SUMMARY

To examine the role of bacteria in the life history of a temporary pond, nine physiological groups of bacteria were determined by plate counts and enrichment culture techniques in pond water, in soil from the pond basin, and in soil from the pinewoods area surrounding the basin. Sampling was begun when the pond was dry and continued through the period when it contained water, into the next dry period. Low counts were obtained for sulphur-, ammonia-, and nitrite-oxidizing autotrophs; iron-oxidizing autotrophs were not detected. This indicates that bacteria did not make a major contribution to the ecosystem as primary producers. Soil samples contained millions to hundreds of millions (10^6–10^8) of aerobic nitrogen-fixers and millions to tens of millions (10^6–10^7) of urea-utilizing organisms per gramme, suggesting that they may be of significance in the nitrogen cycle in the ecosystem. Hundreds of thousands to tens of millions (10^5–10^7) of cellulose-decomposers, and tens of millions to hundreds of millions (10^7–10^8) of aerobic and of anaerobic heterotrophs were present per gramme in soil samples. The several heterotrophic types were also present in pond water in maximum numbers ranging from tens of thousands per millitre for nitrogen-fixers to millions per millilitre for anaerobic heterotrophs. As the pond dried the numbers of bacteria in its water decreased. These data suggest that bacteria in the pond ecosystem play a role in the nitrogen, carbon and energy cycles as decomposers and transformers, as a source of nutrilites and as members of the food chain.

INTRODUCTION

Temporary ponds are bodies of water which dry at least once a year (Shelford, 1913; Kenk, 1949; Rzoska, 1961). Their characteristic biota has been enumerated (Shelford, 1913; Jewell, 1927; Rzoska, 1961, and others), but we are aware of no studies dealing with the role of bacteria in such bodies of water. We examined the bacterial flora of one such pond from its dry stage through a cycle of filling and drying. The pond studied was chosen in part because some data were available about its biota (Moore, 1959, 1963) and the chemical composition of its water and soil (Margavio, 1964). The pond was notable for its lack of primary producers (Margavio, 1964), and it was therefore of interest to examine the bacterial flora to determine its role in the cycling of potential food materials.

The pond is located near Florenville, St Tammany Parish, R 14 East, T 7 South, section 19 (Slidell quadrangle), Louisiana, U.S.A. It was designated Station No. 12 by Margavio (1964). When flooded, the pond covers an area of 7350 m.^{2}. Its average depth is 75 cm. with a maximum depth of 100 cm. at the centre. The basin contains

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water oak, sweet gum and black gum. Cattle and hogs which roam the area contribute nitrogen from their excreta, and interrupt succession of the basin to dry land by their wallowing. Leaf litter also contributes organic matter to the basin area.

METHODS

Sampling. Samples were taken over the period from 8 November 1964 to 8 June 1965. The pond's fill history during this period is shown in Fig. 1. Water was first noted in the basin on 27 November 1964; the pond contained water until late April 1965. The first samples were taken when the pond had been dry for 2 weeks. Additional samples were taken when the pond was drying and the last samples were taken when the pond had been dry for 6 weeks.

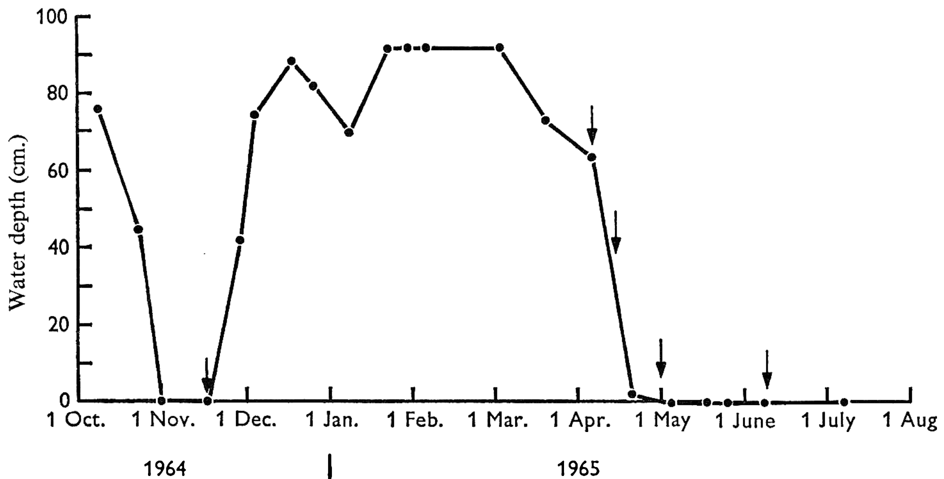

Fig. 1. Fill history of Station No. 12 from October, 1964 to August, 1965. Arrows indicate sampling dates.

Soil samples were taken from pinewood soil in the immediate vicinity of the basin and from the basin proper after the water had receded. An area of forest was cleared of litter and a trenching tool was used to remove a soil sample approximately 3 in. in depth. The sample was placed in a sterile polyethylene bag and the bag was sealed. When the pond contained water, samples were obtained from the approximate centre by submerging a closed sterile polyethylene bag just beneath the surface. The bag was opened, allowed to fill and it was then sealed. Samples were brought to the laboratory and plating was begun immediately.

Microbiological. One-gramme samples of soil were suspended in 99·0 ml. sterile distilled water and homogenized in a Waring blendor. Serial tenfold dilutions were prepared from this suspension with 0·85 % NaCl as diluent. When enumerating autotrophs, 1·0 g. soil was suspended in water and centrifuged at 1200 *g* for 30 min. at 20°. The sediment, containing autotrophs (Mayeaux, 1961), was suspended in 99·0 ml. water, homogenized and diluted in saline. Water samples were diluted directly in sterile saline. At least three plates were prepared for each dilution plated. The time between sampling and completion of plating was about 3·5 hr.

Soil extract. Soil extract was prepared by suspending 500 g. soil from the basin in

1000 ml. distilled water. The suspension was autoclaved and the supernatant fluid passed through a Millipore filter. The liquid portion was used as a medium component (10%, v/v) for heterotrophs, nitrogen fixers and urea decomposers. The pH value of soil extract ranged from pH 4·4 to 5·6.

Media. Thornton's (1922) medium was used for aerobic heterotrophs. Anaerobes were plated on Difco nutrient agar. Tubes of agar were held in boiling water for 5 min. before plating to remove dissolved oxygen. Nitrogen fixers were cultivated on Burk's nitrogen-free medium (Wilson & Knight, 1952).

For cellulose decomposers the basal salts agar of Waksman & Carey (1926) was used as a base layer in plates. Acid-hydrolyzed cellulose agar (Sarkaris & Fazal-Ud-Din, 1933) was added as a second layer. Urea soil extract agar (Allen, 1959) was used for urea-utilizing organisms.

The basal salts medium of Stephenson (1949), as modified by Mayeaux (1961), was used to cultivate ammonia oxidizers; the medium contained (g./l. distilled water): K_2HPO_4, 0·75; KH_2PO_4, 0·25; $FeSO_4.7H_2O$, 0·01; $MgSO_4.7H_2O$, 0·03; $MnSO_4.H_2O$, 0·01; $(NH_4)_2SO_4$, 1·0; saturated phenol red solution, 0·20 ml. The medium was prepared at double strength and diluted with an equal volume of solidifying agent when preparing plates. Preliminary experiments showed that higher counts were obtained when Ionagar No. 2 was used as solidifying agent than when washed agar or silica gel was used. Ionagar No. 2 (Consolidated Laboratories, Inc., Box 234, Chicago Heights, Illinois, U.S.A.) was prepared according to Bechtle & Scheer (1958). This medium was also used for nitrite oxidizers, but with 1·0 g. $NaNO_2$ instead of $(NH_4)_2SO_4$.

The mineral base medium of Colmer (1962) and the 9K medium of Silverman & Lundgren (1959) were both used for autotrophic iron oxidizers; washed agar (Colmer, 1962) or Ionagar No. 2 was used to prepare plates. The medium of Waksman (1922), as modified by Colmer (1962), was used to cultivate autotrophic sulphur oxidizers; washed agar on Ionagar No. 2 was used for plates.

Enrichment cultures. For ammonia oxidizers, 100 ml. of a 1% (w/v) suspension of soil was homogenized in a blendor and transferred to a sterile Erlenmeyer flask. For pond water a 100 ml. sample was placed directly in a flask; 10 ml. of 10% (w/v) filter-sterilized $(NH_4)_2SO_4$ were added and the flask incubated on a reciprocal shaker at room temperature (25°). Additional 10 ml. portions of $(NH_4)_2SO_4$ solution were added daily for 3 days. After 12 days cultures were removed from the shaker. Cultures prepared from soil samples were centrifuged, the sediment homogenized, diluted and plated to determine whether growth had occurred. Cultures prepared from water samples were diluted directly from enrichment cultures. Enrichment cultures for nitrite oxidizers and iron oxidizers were prepared in the same manner, substituting 10% (w/v) solutions of $NaNO_2$ or 10% $FeSO_4.7H_2O$, respectively, for $(NH_4)_2SO_4$.

Spore counts. Soil suspensions were heated at 85° for 10 min. and appropriate dilutions plated on Thornton's agar.

Incubation. All cultures were incubated at room temperature (25°). Anaerobes were incubated under illuminating (petroleum) gas in a vacuum desiccator. A reduced methylene-blue solution was placed in the desiccator to serve as an indicator of anaerobic conditions (McLung & Lindberg, 1957).

Colonies were counted after 2–10 days of incubation, depending on the rate of growth of the several types of organisms.

RESULTS

The smallest numbers of organisms which could be quantitated accurately from the dilutions plated were 1×10^5 and 1×10^3 for soil and water samples, respectively, although numbers as small as one-tenth of those quantities should have yielded colonies on plates. Numbers of organisms below the sensitivity of the methods used should not play an important role in the life history of the pond.

Table 1. *Sample dates, fill history of the pond and numbers of aerobic nitrogen-fixing organisms in soil and water samples*

Sample no.*	Sample date	Fill history of pond	Colony-forming units from: Pond water	Basin soil	Pinewood soil
1	20 Nov. 1964	Dry 2 weeks	.	1.8×10^8/g.	3.4×10^8/g.
2	4 Apr. 1965	Drying	2.6×10^4/ml.	3.4×10^7/g.	4.5×10^7/g.
3	13 Apr. 1965	Drying	None	n.d.†	n.d.†
4	1 May 1965	Dry 1 week	.	3.3×10^6/g.	7.9×10^6/g.
5	8 June 1965	Dry 6 weeks	.	7.6×10^7/g.	7.8×10^7/g.

* In subsequent tables reference to sample data and fill history of the pond will be made by these sample numbers.

† The notation n.d. in this Table and in subsequent Tables indicates that no determination was made.

Table 2. *Numbers of aerobic heterotrophs and anaerobic heterotrophs in soil and water samples*

Sample no.	Aerobic heterotrophs colony-forming units from: Pond water	Basin soil	Pinewood soil	Anaerobic heterotrophs colony-forming units from: Pond water	Basin soil	Pinewood soil
1	.	5.1×10^8/g.	4.2×10^7/g.	.	5.3×10^8/g.	2.8×10^8/g.
2	1.6×10^5/ml.	4.2×10^7/g.	2.5×10^7/g.	4.3×10^6/ml.	6.9×10^7/g.	2.0×10^7/g.
3	None	n.d.	n.d.	2.0×10^5/ml.	n.d.	n.d.
4	.	2.6×10^7/g.	1.7×10^7/g.	.	2.8×10^8/g.	2.5×10^8/g.
5	.	7.9×10^8/g.	1.5×10^8/g.	.	6.8×10^8/g.	9.4×10^8/g.

Basin and pinewood soil yielded 10^6–10^8 aerobic nitrogen-fixing organisms/g. soil (Table 1). Pond water contained 2.6×10^4 nitrogen-fixers/ml. As the pond dried the number in the water decreased below the sensitivity of the method.

Heterotrophs were the largest physiological group isolated from the pond environment. More anaerobes than aerobes were present in pond water, and numbers of both types decreased as the pond dried (Table 2). Soil samples contained 10^7–10^8 organisms/g. Each type decreased over the period when the pond was drying and then increased. Anaerobes increased more rapidly than aerobes after the area was dry. Results of heat-shock experiments indicated that approximately 1·5% of the population of aerobic heterotrophs was composed of heat-resistant (spore) forms.

Bacteria capable of decomposing cellulose were present in pond water and in soil samples (Table 3). Numbers in water decreased as the pond dried. Soil samples taken in the spring yielded fewer cellulose-decomposers than samples taken in the autumn.

Urea-utilizing organisms were present in pond water but decreased below the limits of sensitivity of the method as drying progressed (Table 3). At each sampling interval,

basin and pinewood soil samples contained approximately the same numbers of urea-using bacteria. Numbers of these organisms in soil samples increased approximately 15-fold when the pond contained water. A flora of 10^6–10^7/g. soil appeared to be characteristic of soil when the pond was dry.

Sulphur-, ammonia-, or nitrite-oxidizing bacteria were detected in only one group of samples, taken when the area had been dry for 6 weeks (Table 4); nitrite-oxidizing organisms were found only in soil samples from the area outside the pond's perimeter. Enrichment cultures (Table 5) indicated that ammonia-oxidizing organisms were present in all samples, but nitrite-oxidizing organisms were present only in soil samples from outside the area of the basin. Enrichment cultures were not used for sulphur-oxidizers. Iron-oxidizing organisms were not detected in any samples by plating or enrichment techniques.

Table 3. *Numbers of cellulose-decomposers and urea-utilizing organisms in soil and water samples*

	Cellulose-decomposers colony-forming units from			Urea-utilizing organisms colony-forming units from		
Sample no.	Pond water	Basin soil	Pinewood soil	Pond water	Basin soil	Pinewood soil
1	.	$8 \cdot 7 \times 10^7$/g.	$4 \cdot 6 \times 10^6$/g.	.	$2 \cdot 0 \times 10^6$/g.	$4 \cdot 6 \times 10^6$/g.
2	$9 \cdot 7 \times 10^5$/ml.	$4 \cdot 9 \times 10^6$/g.	$4 \cdot 8 \times 10^6$/g.	$8 \cdot 6 \times 10^5$/ml.	$9 \cdot 3 \times 10^7$/g.	$9 \cdot 3 \times 10^7$/g.
3	$7 \cdot 5 \times 10^4$/ml.	n.d.	n.d.	None	n.d.	n.d.
4	.	$5 \cdot 5 \times 10^6$/g.	$9 \cdot 2 \times 10^6$/g.	.	$3 \cdot 4 \times 10^6$/g.	$6 \cdot 3 \times 10^6$/g.
5	.	$1 \cdot 9 \times 10^5$/g.	$7 \cdot 5 \times 10^5$/g.	.	$3 \cdot 0 \times 10^7$/g.	$1 \cdot 3 \times 10^7$/g.

Table 4. *Numbers of sulphur-oxidizing, ammonia-oxidizing and nitrite-oxidizing autotrophs in samples collected 8 June 1965, when the pond had been dry for 6 weeks.*

	Colony-forming units from	
Type of Type of organism	Basin soil	Pinewood soil
Sulphur-oxidizing	$7 \cdot 0 \times 10^5$/g.	$1 \cdot 7 \times 10^5$/g.
Ammonia-oxidizing	$2 \cdot 1 \times 10^6$/g.	$2 \cdot 4 \times 10^6$/g.
Nitrite-oxidizing	None	$3 \cdot 4 \times 10^4$/g.

Table 5. *Results obtained from enrichment cultures.*

	Growth in cultures prepared in		
Type of organism	Pond water	Basin soil	Pinewood soil
Ammonia-oxidizing	+*	+	+
Nitrite-oxidizing	−	−	+
Iron-oxidizing	−	−	−

* + indicates that growth was observed on plates prepared from enrichment cultures; − indicates that growth was not observed on such plates.

DISCUSSION

In comparison with other reports (Waksman, 1952), large numbers of nitrogen fixing organisms were detected in this pond ecosystem. Margavio (1964) reported values of pH 4·9–5·5 for pond water and 4·8–4·9 for soil of Station No. 12. Aerobic

nitrogen-fixers seldom occur below pH 6·0, although Starkey & De (1939) reported that *Azotobacter indicum* grew below pH 6·0 and Ruinen (1956) observed that members of the genus *Beijerinckia* grew at pH 3·0. We did not determine numbers of anaerobic nitrogen-fixers.

In October 1964 hurricane Hilda brought about complete leaf fall. Over the period of this study, estimates of leaf litter ranged from 1186 to 1603 g./m.^{2}, and basin and pinewoods soils averaged 32 % and 13·3 % organic matter, respectively. The amount of cellulose and other organic materials undoubtedly contributed to the populations of cellulose-decomposers and other heterotrophs in the pond area. Anaerobic heterotrophs and cellulose decomposers were the only groups present in pond water in sufficient numbers to be quantitated in the latter stages of drying (sample 3, Tables 1–3).

Urea-utilizing organisms were present in significant numbers. Their numbers may have been enhanced by urea from animal sources. These organisms may contribute ammonia to the environment, which may be significant at the level of micro-environment. Margavio (1964) obtained values of 0·65–2·9 p.p.m. for ammonium in pond water from Station No. 12. Ammonia-oxidizing organisms were present in all samples tested (Table 5), but not in significant numbers except for samples taken after the area had been dry for 6 weeks (Table 4). It is therefore difficult to evaluate the role of ammonia-oxidizers in this ecosystem.

Absence of iron-oxidizers was not surprising since soil and water samples contained only 0·48–6·0 p.p.m. of ferrous iron. Except for soil samples taken 8 June 1965, sulphur-oxidizers were evidently present in lower numbers than could be detected by our techniques; this correlates with Margavio's (1964) failure to detect sulphur in the pond environment. The reason for the increase in sulphur-oxidizers after the pond dried is unknown.

As the pond dried the bacterial content of the water decreased. This was probably due to adsorption of bacteria to organic particles which sedimented, and to ingestion of bacteria by organisms higher in the food chain. No other correlation was detected between numbers of bacteria and the stage of the pond's fill history through the single cycle examined.

A complete picture of the role of micro-organisms in the natural history of the pond must await further data, particularly about fungi and protozoa. Additional samples should be taken, particularly during the periods when the pond is filling. However, the numbers of autotrophic organisms indicate that bacteria do not make a significant contribution as primary producers. Aerobic nitrogen-fixers and urea-decomposers may play a significant role in the nitrogen cycle. Together with cellulose-utilizing organisms and other heterotrophs, they may also play a major role in the cycling of carbon and energy in the ecosystem: as decomposers and transformers of organic material, as a source of nutrilites in the pond and in the soil which may be washed into the pond as it fills, and as a primary source of food for protozoa and plankton.

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Resistance to Nisin and Production of Nisin-Inactivating Enzymes by Several Bacillus Species

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SUMMARY

The resistance to nisin was examined for the vegetative forms and the endospores of 14 strains of 9 species of the genus *Bacillus*. Bacilli, endospores and culture filtrates were tested for ability to inactivate nisin. Marked anti-nisin activity was detected in extracts of bacilli and endospores of organisms which have a lytic mechanism for rupture of the spore coat (type L); little or no activity was observed in extracts from organisms which rupture the spore coat mechanically (type M). No significant extracellular activity was detected, except after the autolysis of bacilli. When organisms were cultivated in the presence of nisin (50 Reading units/ml.; Berridge, 1949) the yield of organisms and the specific activity of the extracts was decreased; evidence to explain these observations is presented. No quantitative correlation was observed between the production of anti-nisin activity and the resistance of vegetative forms.

The anti-nisin activity of cell-free extracts of bacilli of two species (*Bacillus cereus* and *B. polymyxa*) was studied further; there was evidence that the activity was probably enzymic. A preliminary study of the properties of the enzymes from these organisms was made. It was found that the anti-nisin enzymes of two strains of *B. cereus* differed from the lytic enzymes previously described (Strange & Dark, 1957*a*, *b*). The anti-nisin enzymes had no effect on polymyxin, gramicidin or bactracin but inactivated subtilin. Proteolytic activity was not observed in the preparations.

INTRODUCTION

Nisinase, an enzyme capable of destroying the polypeptide antibiotic nisin, has been shown in nisin-resistant strains of streptococci (Alifax & Chevalier, 1962; Galesloot, 1956; Lipinska & Strzalkowska, 1959), and in strains of *Staphylococcus aureus* (Carlson & Bauer, 1957). In a restricted range of substrate specificity tests the nisinase of *Streptococcus thermophilus* was shown to be active against nisin but not against some other antibodies tested (Alifax & Chevalier, 1962). Gould & Hurst (1962) and Gould (1964) showed that the nisin resistance of endospores of certain Bacillus species was related to the post-germination mechanism for the rupture of the spore-coat. They showed that spores which had a lytic mechanism for rupture of the spore-coat (type L) contained a nisin-inactivator which was not detectable in spores which ruptured the spore-coat mechanically (type M), and they showed that type L spores had a higher resistance to nisin than had type M spores. They also showed that there was little difference in the resistance of spores and vegetative forms of type L organisms, but that the vegetative forms of spore type M organisms were often 20 times

more resistant than were the corresponding endospores. The present paper describes work to determine whether the natural resistance of vegetative forms of species of Bacillus is related to the production of constitutive or inducible enzymes capable of inactivating nisin. Factors which affect the production of the nisin-inactivating enzymes of *Bacillus cereus* and *B. polymyxa*, and some of the properties of these enzymes, were examined.

METHODS

Organisms. The species and strains of the organisms used in this work are given in Table 1. A culture of *Bacillus cereus* (PX) was also used in some experiments. All the organisms conformed to the descriptions given by Smith, Gordon & Clark (1952). Stock cultures were maintained on nutrient agar (Oxoid no. 1) at 4° and were subcultured monthly.

Resistance of organisms to nisin. The resistance of organisms to nisin was determined by plating 0·02 ml. of a standardized washed suspension of vegetative forms, or of heat-shocked (85° for 15 min) endospores, on the air-dried surface of plates of glucose tryptone agar (pH 6·8) containing from 0 to 100 Reading units (r.u.) nisin/ml. (For Reading units, see later; Berridge, 1949). The highest concentration of nisin which permitted macroscopic growth after incubation for 18 hr was taken as the measure of resistance of the organism. All tests, which were repeated on three separate occasions, were done in duplicate and the mean degree of resistance recorded.

Production of cell-free extracts. Conical flasks (1 l.), each containing 250 ml. Tryptone Soya (TS) broth (Oxoid; pH 6·8) were inoculated to 1% (v/v) with a 6 hr shake culture of the organism in TS broth. The flasks were shaken for 18 hr at the appropriate temperature (Table 2) in a water bath. Organisms were harvested by centrifugation, washed by centrifugation four times, each with 50 ml. sterile buffer (0·1 M-citric acid + 0·2 M-Na_2HPO_4; pH 7·0; McIlvaine, 1921), and were then suspended in 10 ml. of the same buffer to give a thick suspension. Acetone powders were prepared at −20° according to the method of Gunsalus (1954). The yield of acetone powder was determined for each organism and a 0·5% (w/v) suspension of the powder in McIlvaine's buffer (pH 7·0) was shaken in a stoppered flask for 5 days at 2° on a Microid flask shaker. Prolonged extraction was required to obtain an active extract from the acetone powders. Insoluble material was then removed by centrifugation at 20,000 *g* for 15 min. and the supernatant fluid used to assay its anti-nisin activity.

When organisms were grown in the presence of nisin, the cultures were incubated for 6 hr before adding to each flask 2·5 ml. of a solution of 5000 r.u. nisin/ml. of 0·02 N-HCl; incubation was continued for a further 12 hr and the organisms then harvested. Acetone powders produced from the washed organisms were extracted with buffer by the procedure outlined above.

In later experiments the acetone powder method was replaced by an autolytic one. Organisms were suspended in McIlvaine buffer (0·05 M-citric acid + 0·1 M-Na_2HPO_4; pH 6·5), to a concentration equivalent to about 50 mg. dry wt./ml, and incubated for 1 hr at 45° in the presence of toluene (2·5%, v/v). After incubation, the autolysate was cooled to 1°, centrifuged at 20,000 *g* for 15 min. and the supernatant fluid used to assay anti-nisin activity. A more active preparation was obtained by the autolytic method than by extraction of acetone-dried organisms.

Culture filtrates. The supernatant culture fluids after centrifugation of cultures to

deposit organisms were sterilized by Seitz filtration and adjusted to pH 7·0 by dissolving one phosphate buffer tablet (Edward Gurr Ltd.) in each 100 ml. filtrate. This solution was then used for the determination of anti-nisin activity.

Preparation of spore extracts. Slopes of 'sporulation agar' (nutrient agar re-inforced with 0·1 % glucose + 0·04 % anhydrous $MgSO_4$) in 16 oz. medical flat bottles were inoculated with 5 ml. of an 18 hr shake culture in TS broth. The inoculum suspension was spread over the surface of the agar and the excess decanted. Slopes were incubated for 8 days, or until sporulation and lysis of the sporangia was complete. The spores were then washed from the surface with sterile distilled water, washed exhaustively with water by centrifugation, the upper layer of the pellet being discarded at each washing to remove residues of vegetative forms. Spores were extracted by shaking in a Mickle tissue disintegrator (H. Mickle Ltd., Gomshall, Surrey) for 2 hr at 2° with McIlvaine buffer (pH 7·0) in the presence of ballotini beads (grade 14). The ballotini and spore debris were removed by filtration through sintered glass filters (no. 2 porosity) followed by centrifugation at 20,000 *g* for 15 min. The supernatant fluid was used for the assay of its anti-nisin activity.

Microscopic examinations. TS broth cultures of the organisms were stained with aqueous fuchsin (Smith *et al.* 1952) and examined for the presence of endospores. The extent of sporulation was determined by counting the number of sporulated organisms per field of view and relating this to the total number of organisms per field. Release of mature endospores was observed by phase-contrast microscopy.

Assay of anti-nisin activity. Anti-nisin activity was measured by incubating suitable volumes (0·1–4·0 ml.) of cell-free extract, or of culture filtrate, with 1 ml. of a solution of nisin (5000 r.u./ml. 0·02 N-HCl), the total volume being adjusted to 9·0 ml. with McIlvaine buffer (pH 7·0). Controls were prepared at the same time and consisted of buffered substrate alone, or of buffered substrate with autoclaved cell-free extract equal in volume to the extract used in the test. The incubated reaction mixtures were at pH 7·0 ± 0·1. Samples (0·9 ml.) removed immediately after preparation of the reaction mixture and at intervals during the incubation period of 6 hr were pipetted into 0·1 ml. of 2 N-HCl to bring to pH 2·0. After mixing, the acidified samples were heated for 5 min. in a water bath at 100° to obtain maximal recovery of nisin, cooled and stored at 2° until required for assay of residual nisin.

The acidified samples were diluted in 0·02 N-HCl by using a 'Clinical Diluter' (Camlab Glass Ltd., Cambridge) to give solutions containing between 2·0 and 10·0 r.u. nisin/ml. Residual nisin was determined by the agar diffusion method of Tramer & Fowler (1964), by using large glass plates (12 in. × 12 in. (30·5 cm.) internal dimensions; each plate required 300 ml. medium to give a depth of about 3 mm.). Standard solutions and test samples were dispensed on each plate, four wells being used for each sample, to allow for variation in diffusion of the antibiotic; the plates were incubated for 18 hr at 30°.

The diameters of the zones of inhibition were measured after projection (Jarvis, 1966) and the concentrations of residual nisin computed from the graph of projected zone diameter plotted against $\log_{10}$ nisin concentration in the standards. The quantity of nisin destroyed was calculated from the difference between the nisin concentrations before and after incubation of the test samples, due allowance being made for loss of activity of the nisin in the controls (about 10 %) and for the dilution factor. This method of assay was reproducible to within ± 10 %.

Units of activity of nisin and of the enzymes. The unit of activity of nisin as defined by Tramer & Fowler (1964) is the activity exhibited by 1·0 μg. of a standard batch of commercial nisin (Nisaplin; Aplin and Barrett Ltd., Trowbridge, Wilts.) and is reputed to be equivalent to the previously defined Reading unit (r.u.: Berridge, 1949). The unit of enzymic activity (e.u.) is an arbitrary unit defined as that amount of enzyme which will inactivate 1000 r.u. nisin in 6 hr at pH 7·0 and at the optimum temperature for the organism from which the enzyme was extracted. It is not possible at this stage to define the activity more precisely, as recommended by the Commission on Enzymes of the International Union of Biochemistry (Report, 1961).

Growth curves. The extinction of TS broth shake-flask cultures was determined after various periods of incubation and growth curves plotted against time. Where necessary, the culture was diluted in TS broth; extinction measurements were made with an EEL Spectra absorptiometer (Evans Electroselenium Ltd., Halstead, Essex) at 650 mμ with a 10 mm. cuvette.

Dialysis of cell-free extracts. Visking dialysis tubing (Hudes Merchandising Co. Ltd., London) was used for dialysis experiments. Before use the tubing was boiled for 30 min. in glass-distilled water and rinsed in six changes of glass-distilled water. Cell-free extracts in McIlvaine buffer (pH 7·0) were dialysed against isomolar buffer or distilled water for up to 48 hr at 2°. The dialysis residue was used for assays of enzyme in the presence of buffered solutions of cations.

Specificity of action of enzyme preparations. The enzyme preparations were tested for ability to hydrolyse casein by pipetting buffered cell-free extract into wells cut in plates of casein agar (10 ml. melted 3% (w/v), Ionagar no. 2, tempered to 50°, mixed with an equal volume of tempered skim milk at the same temperature, poured into Petri dishes and allowed to set). After incubation at 30° for 18 hr the plates were examined for cleared zones around the wells.

Activity against bovine serum albumen (Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex) and against gelatin was assessed by formol titration. The reaction mixtures consisted of 1·0 ml. cell-free extract and 50 ml. 1% (w/v) protein solution adjusted to pH 7·0. After incubation for periods up to 72 hr, samples were assayed for increase in formol titration. Substrate and enzyme controls were run simultaneously and all reactions were compared with the activity of 1·0 ml. of a solution of Bacto-trypsin 1% (Difco Ltd.).

The renneting action of buffered cell-free extracts (1·0 ml.) was determined against 10 ml. freshly reconstituted skim milk in the presence of 0·02 M-$CaCl_2$, at pH 6·0 and 7·0. Controls of substrate and of substrate + rennet were incubated simultaneously at 30°.

The preparations were tested against the polypeptide antibiotics bacitracin, polymyxin (both from the Wellcome Research Laboratories, Beckenham, Kent), gramicidin (Koch-Light Laboratories Ltd., Colnbrook, Bucks) and subtilin (kindly supplied by Dr J. C. Lewis, U.S.D.A., Albany 10, California, U.S.A.). Residual antibiotic was determined on the pre- and post-incubation samples by agar diffusion assay against *Micrococcus flavus*.

Cell-free extracts of *Bacillus cereus* (PX and NCIB 3329) were assayed against isolated cell-wall preparations which had been made from vegetative forms of the same organisms as described by Salton & Horne (1951), and freeze dried. Lytic activity was measured by observing the decrease in extinction of a cell-wall suspension when

incubated with enzyme preparation in the presence of Co^{2+} (Strange & Dark, 1957*b*). Although this method gave an indication of lytic activity it was not very satisfactory.

Electrophoresis of cell-free extracts. Samples of buffered cell-free extract were freeze-dried and redissolved in 1/10 the original volume of distilled water. Electrophoresis was done on cellulose acetate, 12 cm. × 2·5 cm. (Oxoid, Ltd.) in a Kohn horizontal electrophoresis tank (Shandon Scientific Co. Ltd., London) with barbitone buffer (pH 8·6, $\lambda = 0{\cdot}05$), acetate buffer (pH 4·0, $\lambda = 0{\cdot}05$) and tris-EDTA buffer (pH 7·0; Smith, 1960). For all experiments a potential of 2·5 V./cm. strip-length was used. Protein bands were stained with Ponceau S according to the method of Kohn (1958).

RESULTS

Resistance to nisin of vegetative forms and endospores of Bacillus species

The mean measures of resistance are presented in Table 1. The first four organisms have an enzymic mechanism for post-germination rupture of the spore-coat (type L) and these endospores were more resistant to nisin than were the endospores of the other organisms, which split the spore-coat mechanically (type M). There was little difference between the degrees of resistance of the vegetative forms of the two groups, with the exception of the thermophilic *Bacillus stearothermophilus* which was highly sensitive to nisin. Although the degrees of resistance for spore coat-splitting (type M) strains were slightly higher than those reported by Gould (1964) the results show the same general pattern. The difference was probably the result of differences in technique.

Table 1. *Resistance of Bacillus species to nisin*

Organism	Strain	Minimum inhibitory concentration of nisin (r.u./ml.) to prevent colony formation by	
		Vegetative inoculum	Spore inoculum
Spore coat type L*			
Bacillus cereus	NCIB 3329	> 100	100
B. cereus var. *mycoides*	NCIB 7586	> 100	75
B. polymyxa	NCIB 8094	> 100	50
B. megaterium	NCIB 6005	75	25
Spore coat type M†			
B. subtilis	CN 665	> 100	6
B. subtilis	NCIB 8739	75	3
B. subtilis	NCIB 8057	> 100	6
B. subtilis	NCFT 129	100	6
B. licheniformis	NCIB 8061	> 100	13
B. licheniformis	NCFT 128	> 100	13
B. coagulans	NCTC 3992	> 100	3
B. pumilis	NCFT 127	> 100	13
B. stearothermophilus	NCIB 8157	3	< 2
B. stearothermophilus	NCIB 8224	6	< 2

* Spore coat type L: enzymic splitting.
† Spore coat type M: mechanical splitting.

Anti-nisin activity of cell-free preparations

Marked ability to inactivate nisin was detected in extracts of type L spores, but none was observed in extracts of type M spores. The anti-nisin activity of cell-free extracts of vegetative forms grown in the presence and absence of nisin (50 r.u./ml. medium) are presented in Table 2. In most instances decreased yields of acetone-dried organisms and smaller specific activities of the extracts resulted from cultivation of the organisms in the presence of nisin. Experiments were later made to explain these observations (see below).

Extracts of nisin-grown organisms from one strain of *Bacillus subtilis* (NCIB 8057) exhibited greater anti-nisin activity than did extracts of the same organisms cultivated in nisin-free medium. Carlson & Bauer (1957) showed that the resistance to nisin of a strain of *B. subtilis* was increased by serial culture in the presence of nisin. It is possible, therefore, that the artificially developed resistance of *B. subtillis* was dependent upon induction of a specific 'nisinase' as is the case with *Streptococcus thermophilus* (Alifax & Chevalier, 1962) and with *Staphylococcus aureus* (Carlson & Bauer, 1957).

Extracellular activity was only observed in filtrates of 18 hr cultures of *Bacillus polymyxa* (0·1 e.u./ml.) and of two strains of *B. subtilis* (0·1 and 0·4 e.u./ml. for strains CN 665 and NCIB 8075, respectively). However, some activity was shown by filtrates of 2-day to 8-day cultures of *B. cereus* (NCIB 3329), *B. cereus* var. *mycoides* and *B. polymyxa*; this is considered to have been due to liberation of intracellular enzymes on autolysis of the organisms. The activity in the filtrates of *B. subtilis* may also have resulted from autolysis, but was possibly due to the production of subtilopeptidase, an extracellular proteolytic enzyme of certain strains of *B. subtilis* (Güntelberg & Ottesen, 1952), which was shown to inactivate nisin (Jarvis, unpublished observation); 100 i.u. subtilopeptidase (B.D.H. Ltd., Poole, Dorset) inactivated about 150 r.u. nisin in 6 hr at pH 7·0. Since all the organisms used in this work showed marked proteolytic activity on laboratory media and since proteolytic activity was observed in many of the culture filtrates this suggests that nisin is unaffected by the extracellular proteolytic enzymes of many Bacillus species.

Effect of nisin on the vegetative forms of Bacillus

Growth curves were prepared with cultures of *Bacillus cereus* (NCIB 3329), *B. polymyxa* and *B. subtilis* (NCIB 8057). The curves in Fig. 1 are typical of those obtained. The addition of nisin to cultures in the lag phase resulted in a prolongation of that phase, whilst addition of nisin during the logarithmic phase caused rapid lysis of the organisms and the introduction of a prolonged secondary lag phase. The degree of lysis and the duration of the secondary lag phase were dependent upon the concentration of nisin added (Fig. 2). After the initial inhibition the cultures grew normally except that an increased tendency to form chains was frequently observed. The nisin resistance of the organisms was not apparently increased. When nisin was added at the end of the logarithmic phase (Fig. 1), little change occurred either in the concentration of the organisms or in the rate of growth. This may have been merely a reflexion of the greater numbers of organisms present at this time. These results are similar to those reported for *Clostridium butyricum* (Ramseier, 1960) and for *Staphylococcus aureus* (Carlson & Bauer, 1957).

Table 2. *Comparison of intracellular production of nisin-inactivating enzymes by Bacillus species*

Organisms were grown in 250 ml. TS broth shake culture for 18 hr. Organisms were harvested by centrifugation, washed with McIlvaine buffer (pH 7·0) and acetone powders were produced at −20°. The acetone-dried organisms were extracted with McIlvaine buffer (pH 7·0), for 5 days at 2°; the extracts were centrifuged at 20,000*g* for 15 min. and the supernatant solution used for assay of anti-nisin activity.

			Yields of acetone-dried preparations and nisin-inactivating activity of extracts of vegetative forms					
			Grown in absence of nisin			Grown in presence of 50 r.u. nisin/ml.		
Organisms	Strain	Temperature of incubation of cultures and assays (T°)	Yield acetone powder (g.)	e.u./g. acetone powder	Total e.u.	Yield acetone powder (g.)	e.u./g. acetone powder	Total e.u.
Spore coat type L								
Bacillus cereus	NCIB 3329	30	1·025	104·2	107·0	0·566	45·0	25·5
B. cereus var. *mycoides*	NCIB 7586	30	1·543	43·8	67·8	0·451	30·4	14·1
B. polymyxa	NCIB 8094	30	1·160	104·0	121·0	0·616	1·8	1·1
B. megaterium	NCIB 6005	37	0·528	60·2	31·8	0·770	0·0	0·0
Spore coat type M								
B. subtilis	CN 665	37	1·850	3·1	5·4	1·444	3·2	4·6
B. subtilis	NCIB 8739	37	0·493	31·0	15·3	0·530	2·0	1·6
B. subtilis	NCIB 8057	37	0·566	25·2	14·3	0·355	54·2	19·3
B. subtilis	NCFT 129	37	0·757	0·0	0·0	0·661	1·4	1·1
B. licheniformis	NCIB 8061	37	0·323	0·0	0·0	0·710	0·0	0·0
B. licheniformis	NCFT 128	37	0·992	0·0	0·0	0·509	0·0	0·0
B. coagulans	NCTC 3992	37	0·531	9·0	4·8	0·861	1·8	1·6
B. pumilis	NCFT 127	37	0·528	0·0	0·0	0·330	0·0	0·0
B. stearothermophilus	NCIB 8157	55	0·511	0·0	0·0	—	—	—
B. stearothermophilus	NCIB 8224	55	0·428	0·0	0·0	—	—	—

Production of the nisin-inactivating enzyme

The anti-nisin activity of *Bacillus cereus* (NCIB 3329 and PX) and *B. polymyxa* was determined for extracts of organisms taken at different stages of growth. No activity was detected until the beginning of sporulation, and the activity reached a peak shortly before the liberation of the endospores (Table 3). These results suggest a similarity between the anti-nisin enzyme and the lytic enzymes previously described by Strange & Dark (1957*a*, *b*). Gould & Hurst (1962) showed that the crude extract from endospores

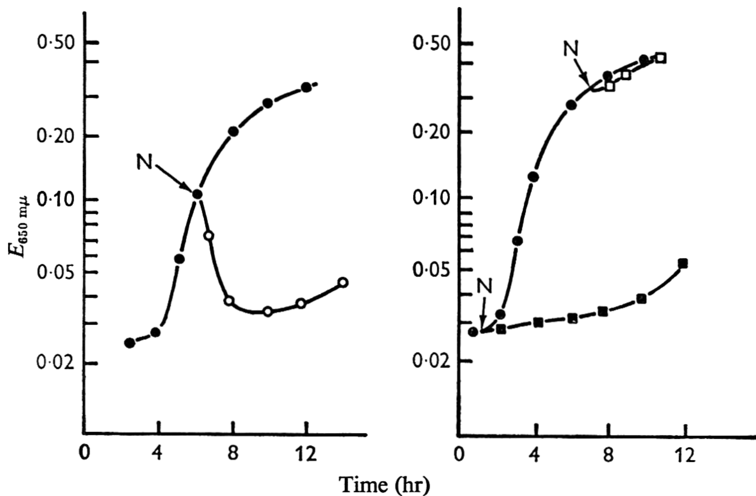


Fig. 1. The effect of nisin on vegetative *Bacillus cereus*. The extinction of TS broth shake cultures was determined at 650 mμ using a 10 mm. cuvette. Nisin was added (N), to a final concentration of 50 r.u./ml. medium, to cultures in the lag phase (■—■), the logarithmic phase (○—○) and at the end of the logarithmic phase (□—□); a nisin-free control (●—●) was incubated simultaneously.

Table 3. *Production of nisin-inactivating enzyme by Bacillus cereus* (PX) *at different stages of growth*

The organisms were grown in TS broth shake culture at 30°. Samples were removed at intervals and extracts were prepared by autolysis of washed organisms at 45°, pH 6·5 for 1 hr. The dry wt. of organisms was determined by drying suitable samples of the suspension of organisms in vacuum at 70° for 18 hr, and the extent of sporulation was determined by microscopy.

Time of incubation (hr)	State of organisms	Nisin-inactivating activity of cell-free extract (e.u./g. dry wt. organism)
6	Non-sporulating vegetative	< 1
18	Sporulation begun	28
24	Sporulation > 90 % complete	1,747
28	Spore-liberation begun	1,828

of *B. cereus* (PX) was active against nisin and Gould & Hitchins (1965) showed that the Strange & Dark enzyme isolated from spores of *B. cereus* (PX) was able to initiate germination in suitably primed spores.

Comparison of the nisin-inactivating and the lytic enzymes of Bacillus cereus

Cell-free extracts of organisms from sporulating cultures of *Bacillus cereus* (NCIB 3329 and PX) were prepared by autolysis at pH 5·5 and pH 7·0 (Strange & Dark, 1957*b*) and by buffer extraction of acetone-dried organisms. The results obtained (Table 4) show that although the crude cell-free extracts were active against isolated cell walls and nisin, the partial purification procedure of Strange & Dark (1957*b*) resulted in total inactivation of the nisin-inactivating enzyme, whilst the wall lytic enzymes remained active. However, the method used for assay of lytic activity was not very satisfactory because of precipitation in the blanks and the low relative activity of the preparations as compared to those reported by Strange & Dark (1957*a*, *b*).

Table 4. *Comparison of nisin-inactivating and cell-wall lytic enzymes of Bacillus cereus*

Extracts of sporulating organisms grown in TS broth shake culture were prepared by buffer extraction of acetone-dried organisms and by autolysis of organisms at pH 5·5 and pH 7·0 (Strange & Dark, 1957*b*). The crude extracts were partially purified by precipitation at pH 3·0 (Strange & Dark, 1957*b*).

		Effect* of enzymes on: Isolated cell walls of *B. cereus*			Nisin †		
Method of extraction	Extraction at pH	Crude extract	pH 3‡ soluble fraction	pH 3† insoluble fraction	Crude extract	pH 3 soluble fraction	pH 3 insoluble fraction
Autolysis §	5·5	++	++	+	+	−	−
Autolysis	7·0	+	+	+	+++	−	−
Acetone powder	5·5	++	+	+	+	−	−
Acetone powder	7·0	+	+	+	+++	−	−

* Activity scored as highly active, +++; active, ++; slightly active, +; inactive, −.

† Assayed at pH 7·0; (Strange & Dark, 1957*b*).

‡ Assayed at pH 4·0; (Strange & Dark, 1957*b*)

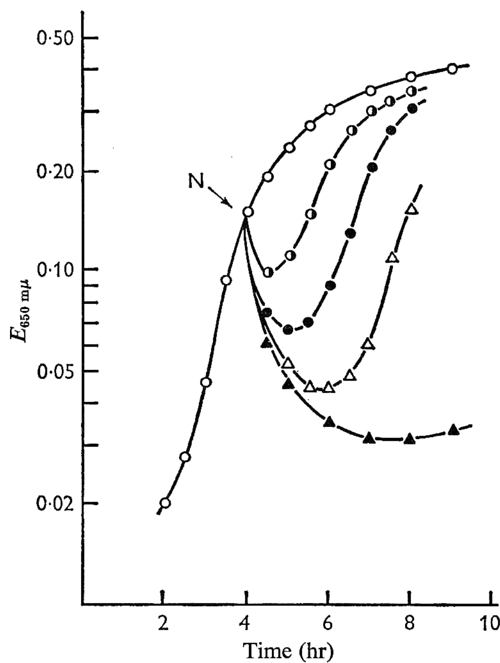
§ Method of autolysis: suspension of organisms in water (about 50 mg./ml.) added to equal volume of McIlvaine buffer +2·5 % (v/v) toluene, and incubated for 1 hr at 37°. Autolysate cooled rapidly to 1°, centrifuged at 20,000 *g* for 15 min. and supernatant fluid stored at −20°.

The crude cell-free extracts of vegetative *Bacillus cereus* (NCIB 3329) and *B. polymyxa* have been shown to be unable to initiate germination of 'primed' spores (Dr G. W. Gould, personal communication, 1966), whilst a preparation of spore-germinating enzyme from *B. cereus* (PX) (Gould & Hitchins, 1965) was unable to inactivate nisin.

Evidence that the inactivation of nisin by cell-free extracts of Bacillus cereus and B. polymyxa is enzymic

In the experiments already described, it was assumed that inactivation of nisin resulted from enzymic action. Some experiments were made to confirm this assumption. The degree of anti-nisin activity was dependent upon the volume of cell-free extract used and upon the time of incubation (Fig. 3). After incubation for 1 hr the amount of nisin inactivated was directly proportional to the volume of extract used but after incubation for 6 hr the rate of reaction had decreased considerably and the decrease in nisin activity was no longer directly proportional to the concentration of

cell-free extract. Where very dilute enzyme preparations were used, e.g. 0·1–1·0 ml. of a 1/100 dilution, the inactivation was proportional to the volume of extract, even after incubation for 18 hr. These observations suggest that the activities reported above in Table 2 may have underestimated the potential activity of the more active preparations. The initial rate of inactivation, obtained from the slope of the tangent to the reaction curve at time 0 hr, is directly proportional to the volume of extract used (Fig. 4). No attempt has been made to determine Michaelis constants for the enzymes.



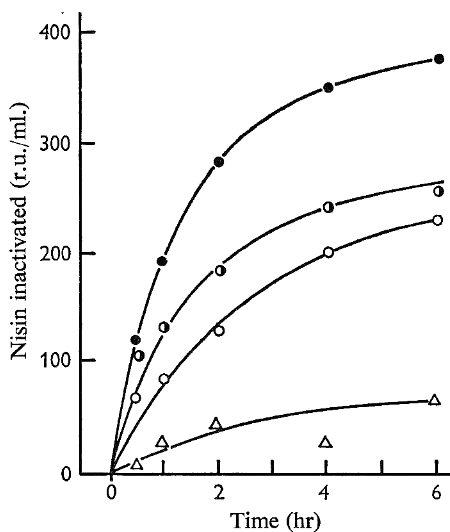


Fig. 2. The effect of nisin concentration on the lysis of logarithmic phase organisms of *Bacillus cereus*. Different volumes of a solution of nisin in 0·02 N-HCl were added (N) to logarithmic phase organisms of *B. cereus* (NCIB 3329) in TS broth shake culture, to a final concentration of 50 r.u./ml. (▲—▲), 25 r.u./ml. (△—△), 12·5 r.u./ml. (●—●), 6·25 r.u./ml. (◑—◑), and 0 r.u./ml. (○—○). Extinction was determined at 650 mμ using a 10 mm. cuvette.

Fig. 3. The effect of time and volume of enzyme preparation on the inactivation of nisin by cell-free extract of *Bacillus polymyxa*. The reaction mixtures consisted of 1·0 ml. of a solution of nisin (5,000 r.u./ml. 0·02 N-HCl), an aliquot of buffered cell-free extract and McIlvaine's buffer (pH 7·0) to 9·0 ml. The curves correspond to cell-free extract used at the following levels: 0·0 ml. (△—△), 1·0 ml. (○—○), 2·0 ml. (◑—◑) and 3·0 ml. (●—●). After incubation at 30° for periods of 0, 0·5, 1, 2, 4 and 6 hr, samples (0·9 ml.) were removed and pipetted into 0·1 ml. 2 N-HCl. The acidified mixtures were boiled for 5 min., cooled and diluted in 0·02 N-HCl for assay of residual nisin.

The preparations were active at low enzyme: substrate ratios, on a dry-weight basis. Experiments were made with partially purified nisin as substrate. At a substrate concentration equivalent to 2·2 mg. nisin A (40 r.u./μg.), 93% inactivation was obtained after incubation for 24 hr at 30° and pH 7·0, with 0·1 ml. (200 μg. dry wt./ml.) cell-free preparation from *Bacillus polymyxa*. In the control series containing either

no enzyme or heat-inactivated enzyme, only a 12% decrease of nisin activity was observed. Whilst the enzyme: substrate ratio in this experiment was only of the order of 1:110 it may be assumed that the partially purified nisin used as substrate contained nisin A, together with other nisins of lower specific activity, so that the actual enzyme: substrate ratio might have been several orders lower than that reported. Marked inactivation of nisin was obtained with *B. cereus* extracts at ratios down to 1:500 (assuming nisin A as substrate).

The nisin-inactivating enzymes were inactivated by heating to 100° for 5 min.; treatment of the preparations at room temperature with ethanol, acetone, trichloracetic acid or sodium tungstate resulted in total loss of anti-nisin activity. These results suggest that the anti-nisin activity was dependent upon the protein moiety of the extracts.

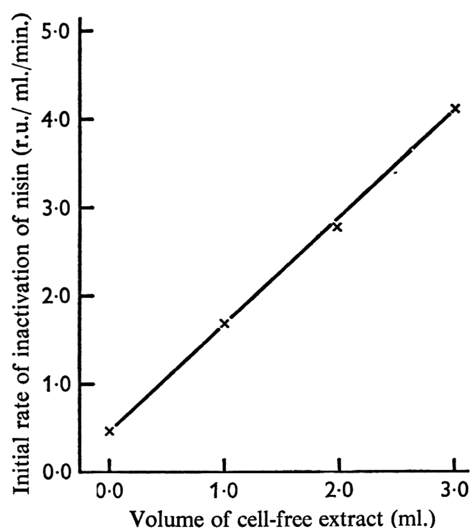


Fig. 4

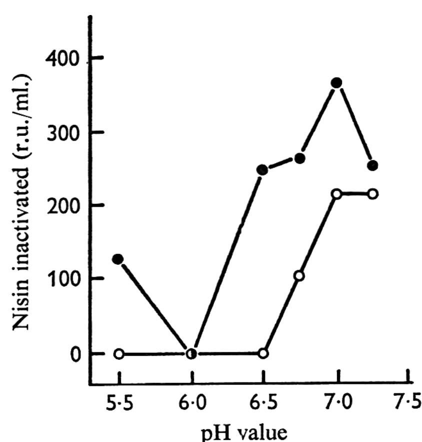


Fig. 5

Fig. 4. The effect of volume of enzyme preparation on the initial rate of inactivation of nisin by cell-free extract of *Bacillus polymyxa*. Initial rates of inactivation of nisin were obtained from results presented in Fig. 3 by constructing the tangent to the reaction curve at time 0 hr. and determining the slope of the tangent. The rate of inactivation of nisin was calculated in terms of r.u. nisin inactivated/ml. reaction mixture/min.

Fig. 5. The effect of pH on the enzymic inactivation of nisin by cell-free extracts of *Bacillus cereus* and *B. polymyxa*. Reaction mixtures consisted of 2·0 ml. buffered (pH 7·0) cell-free extract of *Bacillus cereus* (○—○) or *B. polymyxa* (●—●), 1·0 ml. of a solution of nisin (5000 r.u./ml. 0·02 N-HCl) and 6·0 ml. of McIlvaine's buffer (0·1 M-citric acid+0·2 M-Na_2HPO_4). Controls were prepared from buffered substrate containing 2·0 ml. heat-inactivated preparation. The pH levels of the reaction mixtures were determined on the incubated mixtures. Aliquots (0·9 ml.) were removed before and after incubation at 30° for 6 hr and pipetted into 0·1 ml. 2 N-HCl. After heating for 5 min. at 100°, the samples were diluted in 0·02 N-HCl for assay of residual nisin. The results presented allow for inactivation of nisin (*c.* 10 %) in the controls.

Extracts of both *Bacillus cereus* and *B. polymyxa* exhibited optimal activity around pH 7·0 (Fig. 5), but it was not possible to assess the activity under alkaline conditions since nisin is chemically inactivated under these conditions. There appeared to be a secondary optimum below pH 6·0 with the extract from *B. polymyxa*.

A preliminary investigation of the effect of enzyme inhibitors and cations on the activity of the extracts showed that both preparations required the presence of Ca^{2+}, Co^{2+} and Mg^{2+} for maximal activity. Activity was decreased after prolonged dialysis against distilled water or buffer (pH 7·0) and was restored by adding these cations (Table 5). No differences were observed in the degree of activity of the preparations dialysed against water and those dialysed against isomolar buffer. This shows that the requirement for certain cations was not a function of the complexing effect of the citrate in the McIlvaine buffer. The enzymic destruction of nisin was inhibited to various degrees by the presence of Zn^{2+}, Fe^{2+}, Cu^{2+} and Mn^{2+} and by EDTA, KCN, cysteine, GSH and *p*-chloromercuribenzoate (Table 6). Inhibition by EDTA confirmed the requirement for certain cations; whilst both cysteine and GSH caused a marked decrease in anti-nisin activity, both reagents themselves caused partial inactivation of the substrate. The effect of iodoacetate and of Hg^{2+} could not be determined since the former resulted in total inactivation of the nisin and the latter was toxic to the test organism.

Table 5. *The effect of added cations on the anti-nisin activity of dialysed cell-free extracts of Bacillus cereus* (NCIB *3329*) *and B. polymyxa*

Cell-free extracts were dialysed at 2° for 48 hr against distilled water. Reaction mixtures consisted of 1·0 ml. dialysed enzyme preparation, 0·5 ml. nisin solution (5,000 r.u./ml.), 0·5 ml. of each cation solution (to 0·1 mM where appropriate) and McIlvaine buffer (pH 7·0) to 4·5 ml. Samples (0·9 ml.) were taken immediately after mixing the reagents and after incubation for 6 hr at 30°, and were pipetted into 0·1 ml. 2 N-HCl. The acidified samples were heated for 5 min. at 100° and were diluted in 0·02 N-HCl for assay of residual nisin.

Enzyme preparation	r.u. nisin inactivated by 0·2 ml. enzyme preparation of *B. polymyxa*	*B. cereus*
Dialysis residue alone	215	45
Dialysis residue + Ca^{2+}	355	195
Dialysis residue + Mg^{2+}	317	135
Dialysis residue + Co^{2+}	240	120
Dialysis residue + Ca^{2+} + Mg^{2+}	413	255
Dialysis residue + Ca^{2+} + Co^{2+}	437	339
Dialysis residue + Mg^{2+} + Co^{2+}	427	291
Dialysis residue + Ca^{2+} + Mg^{2+} + Co^{2+}	401	307

Specificity of the anti-nisin enzymes

The cell-free preparations from vegetative *Bacillus cereus* and *B. polymyxa* had no observable effect against casein, gelatin or bovine serum albumin, and neither caused coagulation of skim milk at pH 6·0 or pH 7·0. From these results it is inferred that the nisin-inactivating enzymes are not proteases. The preparations were unable to decrease the potency against *Micrococcus flavus* of the polypeptide antibiotics bacitracin or polymixin B and destruction of gramicidin could not be shown. However, the anti-nisin preparations were active against subtilin and resulted in marked inactivation.

Electrophoresis of preparations from vegetative Bacillus cereus and B. polymyxa

The electrophoretic patterns produced on cellulose acetate were similar for extracts of both *Bacillus cereus* and *B. polymyxa*. The best separation was obtained with tris-EDTA buffer (pH 7·0); under the experimental conditions used four major and several minor fractions were observed. No attempt has yet been made to determine whether more than one of these fractions is active against nisin.

Table 6. *The effect of inhibitors on the anti-nisin activity of cell-free extracts of Bacillus cereus and B. polymyxa*

Reaction mixtures consisted of 1·0 ml. enzyme preparation, 0·5 ml. nisin solution (5000 r.u./ml.), 0·5 ml. inhibitor solution and McIlvaine buffer (pH 7·0) to 4·5 ml. Samples (0·9ml.) were taken immediately after mixing the reagents and after 6 hr incubation at 30°, and were pipetted into 0·1 ml. 2 N-HCl. The acidified samples were heated for 5 min. at 100° and were diluted in 0·02 N-HCl for assay of residual nisin.

Inhibitor	Concentration of inhibitor (mM)	r.u. nisin inactivated by 0·2 ml. enzyme preparation of	
		B. polymyxa	*B. cereus*
None	—	350	182
Mn^{2+}	0·1	308	151
Zn^{2+}	1·0	245	125
Fe^{2+}	1·0	25	0
Cu^{2+}	1·0	0	10
EDTA	1·0	60	0
KCN	1·0	0	93
p-Chloromercuri-benzoate	1·0	280	132
Cysteine	1·0	12	0
GSH	1·0	69	71

DISCUSSION

Three general modes of resistance to antibiotics have been described. The first of these is the production of an enzyme capable of destroying the antibiotic, as in the case of penicillinase (Bondi & Dietz, 1948; Pollock, 1957) and nisinase (Alifax & Chevalier, 1962). The second mode of resistance is due to changes in the cell-wall permeability, e.g. resistance to chloramphenicol (Kushner, 1955) and actinomycin (Polsinelli *et al.* 1964). The third type is the acquisistion of some other mechanism (e.g. modification of the antibiotic-binding capacity of DNA) as reported for chlortetracycline (Saz & Martinez, 1956) and erythromycin (Taubman, Young & Corcoran, 1963).

The production of nisin-inactivating enzymes by certain species of the genus *Bacillus* (*B. cereus*, *B. cereus* var. *mycoides*, *B. polymyxa*, *B. megaterium*) suggests that the mechanism of resistance to nisin by these organisms is dependent upon enzymic destruction of the nisin. Whilst nisin produces inhibition at all stages of growth the effect is more pronounced during the early phases when no intracellular anti-nisin enzyme is detectable. This suggests that the resistance of mature vegetative forms and of endospores of these species may be linked to production of the enzyme. However, since young organisms were able to overcome the inhibitory effect of nisin, the mode

of resistance may be unrelated to the mechanism for production of the enzyme. It is possible that the enzyme is produced by the mature vegetative organisms for some function other than inactivation of nisin; however, the function of the enzyme would appear to differ from that of the lytic enzymes previously described (Strange & Dark, 1957*a*, *b*).

Since the vegetative forms of the Bacillus species examined (except *B. stearothermophilus*) were resistant to similar concentrations of nisin, it appears that a general mechanism of resistance other than production of nisin-inactivating enzyme is prevalent within the genus. It is possible that the anti-nisin enzyme is produced by the sporulating organism and then adsorbed on to, or combined with, the wall of the type L endospore. This would explain the observation that type L endospores, which contain an extractable nisin-inactivating enzyme, are more resistant to nisin than are type M endospores (Gould & Hurst, 1962; Gould, 1964).

It has been reported that nisin is inactivated by treatment with trypsin (Thorpe, 1960) and pancreatin (Heinemann & Williams, 1966). It is not surprising therefore that 'subtilopeptidase' also causes inactivation. However the extracellular proteases from several other Bacillus species appear to be unable to inactivate nisin; this suggests that these enzymes may have a mode of action which differs from that of tryptic-type of enzyme.

The relative decrease of the yield of organisms when cultivated in the presence of nisin is a direct result of nisin-induced lysis and of the secondary lag phase, since in the experiments made nisin was added to cultures in the logarithmic phase and the organisms were harvested after the same total incubation period as organisms grown in the absence of nisin. Furthermore, the lower specific activity of extracts obtained from organisms grown in medium containing nisin, resulted from the secondary lag phase. At the time of harvesting the organisms would be in a relatively less mature state and would have produced less enzyme than those organisms growing in nisin-free medium. The observed decrease in the specific activity of the extracts is consistent with these conclusions.

The results obtained in this work suggest that the anti-nisin action of cell-free extracts of *Bacillus cereus* and *B. polymyxa* is due to enzymic activity. Although the tests of substrate specificity were limited, activity was observed only against nisin and subtilin. Both of these polypeptides contain the S-amino acids β-methyllanthionine and lanthionine in the ratio of 4:1 (Lewis & Snell, 1951; Newton, Abraham & Berridge, 1953), although Cheeseman & Berridge (1959) have since shown a ratio of 3:1 in nisin A. However, the antibiotics differ in their content of other amino acids (Berridge, Newton & Abraham, 1952; Lewis & Snell, 1951; Cheeseman & Berridge, 1959). In view of the similarity in the S-amino acid composition of nisin and subtilin it is possible that lanthionine and β-methylanthionine form part of the active centre of the antibiotics and that the enzyme acts by disrupting this structure.

The properties of the nisin-inactivating enzymes of *Bacillus cereus* and *B. polymyxa* appear to be similar, but no similarities exist between the properties of the nisin-inactivating enzyme of *B. cereus* and the extracellular protease of this organism described by Salter (1959). The crude cell-free extracts of vegetative forms of *B. cereus* and *B. polymyxa* contain at least seven protein components.

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A New Principle for the Determination of Total Bacterial Numbers in Populations Recovered from Aerosols

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SUMMARY

Although the most reliable techniques for determining total bacterial numbers in populations recovered from aerosols are based on radioactive tracers, non-isotopic methods must be used for certain purposes. A tracer technique based on the enzyme galactosidase was developed for the determination of bacterial numbers in samples containing *Escherichia coli* strain B organisms 1 to 2×10^7/ml. By using the [^{14}C]-tracer technique as a reference standard, the enzyme method was shown to be unaffected by the relative humidity at which the aerosol was stored and by the viability of the recovered population and to be scarcely affected by the age of the bacterial cloud. The principle of this method may be applied to other organisms and other suitable enzymes.

INTRODUCTION

Laboratory investigations with pathogenic microbes or simulants can often indicate the importance of aerosols as vehicles for the spread of infections. It is often necessary in laboratory studies to determine total bacterial numbers in populations recovered from aerosols. The concentration of an aerosol and the extent of physical losses due to storage or dilution may then be estimated from total numbers in recovered samples and from the characteristics of the sampling device. The viability of populations recovered from aerosols may also be estimated from the total and viable bacterial numbers in samples. Viability forms a particularly useful measure of the effect of various stresses on survival in the aerosol and may give an indication of the infectivity of a pathogenic microbial aerosol *in vivo*.

Total bacterial numbers in aerosols may be estimated either by light-scatter measurements on the aerosol itself, or by addition of tracers such as dyes, stable bacterial spores or radioactive materials to the suspension from which the aerosol is generated (methods reviewed by Anderson & Cox, 1967). Light-scatter methods on the aerosol (e.g. Ferry, Farr, Rose & Blau, 1951; Dimmick, 1960) have to be calibrated by a tracer method and are susceptible to variations in relative humidity, spray fluids, etc. The dye tracer method (e.g. Ferry *et al.* 1951; Wolfe, 1961) has the disadvantage that the dye may prove toxic to the organism used, especially since concentration occurs through evaporation of water from aerosol droplets. The dye method has the further disadvantage that when aerosols are generated from bacterial suspensions in water or dilute solutions, particles containing dye alone may be smaller than those containing dye + bacteria and thus have different physical properties. In the spore tracer method the presence of an organism additional to the one under examination may complicate

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viability determinations and biochemical reactions. There is evidence that the spore tracer in common use, namely *Bacillus globigii* (*B. subtilis* var. *niger*), suffers a rapid initial death in the aerosol (Anderson, 1966; Cox, 1966). Radioactive tracer techniques, although most reliable, cannot be applied when metabolic processes have to be studied with radiotracers subsequent to collection from the aerosol or in cases where expense or health hazards rule out the use of radiotracers. A tracer technique based on an aerostable enzyme has therefore been developed and this paper illustrates the principle using the β-galactosidase of *Escherichia coli* strain B.

METHODS

Organism and media. Cultures of *Escherichia coli* strain B, were maintained as described by Anderson (1966); likewise the liquid and solid tryptone media and fluids used for the recovery of organisms from aerosols were as described by Anderson (1966).

Growth of organisms. Liquid tryptone medium (10 ml.) supplemented with [U-^{14}C]-D-glucose (about 60 μg./ml.; 1·25 μC/ml.; obtained from the Radiochemical Centre, Amersham, Buckinghamshire) and isopropyl-thio-β-D-galactopyranoside (0·01 mmole/ml.; supplied by Mann Research Laboratories, Inc., New York 6) was inoculated (to 5×10^7 organisms/ml.) with a suspension of *Escherichia coli* strain B. After growth in shake culture (100 ml. flasks at 37° for 16 hr) the suspensions were stored at room temperature for 2–5 hr before use. The washed bacteria contained about 30% of the added radiotracer and hydrolysed about 5 μmoles of *o*-nitrophenol-β-D-galactopyranoside/10^{10} organisms/min. in the test described below. Immediately before the generation of an aerosol the organisms were washed once with water by centrifugation then prepared as an aqueous suspension containing about 3×10^9 organisms/ml.

Generation, storage and recovery of aerosols. Monodisperse aerosols generated in an air-jet atomizer were diluted in a stainless steel tube with a stream of air of controlled water content, to give an atmosphere of the desired relative humidity (Henderson, 1952). Aerosols emerging from the mixing tube could be stored in a rotating stainless steel drum (Goldberg, Watkins, Boerke & Chatigny, 1958). Aerosol samples were collected into phosphate buffer+alginate (Henderson, 1952) fluid by using a sonic impinger (raised Porton impinger of May & Harper, 1957) to yield suspensions containing about 1 to 2×10^7 organisms/ml. Further experimental detail was as described previously (Anderson, 1966).

Determination of radioactivity. Apparatus, methods and materials for the determination of radioactivity by a 'coincidence counting' technique with a liquid scintillator were as described by Anderson & Smith (1965). [^{14}C]-Determinations had a 95% confidence belt of ± 5%.

Determination of the galactosidase content of bacterial populations. The method used was as described by Anderson (1966) except that bacterial samples were incubated with benzene and *o*-nitrophenol-β-D-galactopyranoside for 40 min. instead of 30 min. Reagents and containers were preheated in a water bath before addition of bacterial suspensions.

Determinations of viable numbers. Viability was defined as the ability of an organism to produce a visible colony in 18 hr at 37° on a tryptone agar medium. The culture methods used were as described by Anderson (1966). Colony counts and radioactivity determinations were so arranged that viability estimates had a 95% confidence belt of ± 10% of observed values.

RESULTS AND DISCUSSION

The enzymic activity of the β-galactosidase of populations of *Escherichia coli* strain B, recovered from aerosols has been shown to be similar to that of non-sprayed organisms. A limited number of results indicated that the activity of this enzyme was scarcely affected by variations in the viability of recovered organisms or by the relative humidity or storage time of the aerosol. Populations which had been induced to form β-galactosidase were shown to have similar survival characteristics in the aerosol to normal organisms (Anderson, 1966). If β-galactosidase were quite stable, and if the enzyme activity and total bacterial count of any suspension were known, then the number of bacteria in populations recovered from aerosols generated from such a suspension could be calculated from the observed galactosidase activity of the recovered population. The reliability of the β-galactosidase tracer technique was assessed by simultaneously determining total bacterial numbers in a representative array of samples by both the galactosidase and the [^{14}C]-tracer techniques. In the [^{14}C]-tracer technique (Anderson, 1966) the test organism itself is labelled under its normal growth conditions with a stable, innocuous and easily determined isotope. At each relative humidity value the [^{14}C] content, galactosidase activity and viable count were determined on suspensions taken from the spray pot before and after the experiment, and on samples recovered from aerosols at 1·2 sec., 5 min., 15 min. and 30 min. after generation.

Table 1. *Evidence for the reliability of the galactosidase tracer technique for the determination of total bacterial numbers in populations recovered from aerosols of Escherichia coli strain* B.

Age of aerosol cloud	Mean* (and 95 % fiducial limits of mean) value for the ratio: $\frac{\text{bacterial numbers by galactosidase method}}{\text{bacterial numbers by [}^{14}\text{C]-tracer method}} \times 100$	
Unsprayed control removed from spray pot after each experiment	98·1	(94·6 → 101·6)
1·2 sec.	99·3	(94·1 → 104·6)
5 min.	102·2	(98·5 → 106·0)
15 min.	105·4	(100·9 → 110·0)
30 min.	105·9	(101·1 → 110·8)

* Each value the mean of twenty determinations over a range of relative humidities from 32 to 93 %. This ratio was given the value of 100 % in unsprayed controls removed from the spray pot before each experiment.

Estimates of total bacterial numbers by the galactosidase method were found to be similar to those determined by the [^{14}C] method over a whole range of relative humidity values and aerosol ages. For each bacterial suspension the sample size determined by the galactosidase method was expressed as a percentage of that obtained by the radiotracer method; this ratio was then used in a statistical analysis of results. There was no significant correlation between this ratio and the relative humidity at which the aerosol was held. Table 1 provides evidence that this ratio tended to increase with increasing aerosol age. This increase, which was comparatively trivial, amounted to about 6 % over 30 min. and might be an artifact caused by the increased accessibility

of the enzyme to the test substrate in thoroughly dried bacteria. Combined results of all 80 determinations on samples recovered from aerosols confirmed that there was a small significant correlation (correlation coefficient 0·24; $P = 3\%$) between aerosol age and the ratio of total bacterial numbers determined by the two methods. If the [^{14}C] technique is regarded as a reference standard, bacterial numbers may be obtained within about 2% of true values by dividing observed values obtained by the galactosidase method by a factor of $(1{\cdot}0065 + 0{\cdot}000035\ T_s)$, where T_s is the aerosol age in seconds. Such accuracy is seldom required in aerosol studies. There was no significant correlation between the galactosidase/radiotracer ratio and the viability of recovered populations (80 samples covering a range of viabilities from 2 to 100% with a mean of 30%).

The extremely close agreement between these two tracer methods which depend upon different principles and techniques shows that the galactosidase tracer method is entirely reliable for *Escherichia coli* strain B. The principle of tracing aerosols with enzymes could be applied to other organisms and other enzyme systems, e.g. the high catalase content of many organisms and the sensitivity of assay methods for this enzyme suggests an alternative approach for certain other bacteria.

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The Continuous Culture of Anaerobic Bacteria

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SUMMARY

Modifications to an anaerobic continuous culture apparatus to allow pH control, and pH and Eh measurements, are described. Two anaerobic rumen bacteria were grown under different conditions, but as carbohydrate-limited cultures. The effects of growth rate, pH value and Eh value on yields of bacteria, enzyme activities and fermentation products are described. Optimum bacterial yields per mole of substrate fermented and per mole of ATP presumably formed in the fermentation were variable with the particular bacterium and the substrate, and were high for hexose fermentations. Yields of bacteria varied with growth rate, being lowest at low growth rates. Fermentation products also varied with growth rate and the pH value of the culture in some cases, as did the production of enzymes. Maximum growth rates calculated from batch cultures were in agreement with those found in the continuous cultures.

INTRODUCTION

Some results obtained from continuous cultures of anaerobic rumen bacteria were described by Hobson (1965*a*). We give here some further observations on continuous cultivation of anaerobic bacteria in a chemostat with external pH control.

METHODS

Apparatus. The apparatus described by Hobson (1965*b*) relied for pH control on the buffering action of the medium, but it was mentioned that external pH control had been incorporated in a later modification of the apparatus. The present apparatus, which has been in use for some years, is basically the same as that previously described (Hobson, 1965*b*) with the following modifications (reference is made to Fig. 1, Hobson, 1965*b*, in this description.) The single gas flow-meter (P) has been replaced by a double flow-meter on the 'apparatus' side of the furnace. This enables not only the oxygen-free carbon dioxide from the furnace to be monitored, but also a stream of some other gas, such as oxygen, to be introduced into the gas flow. In place of the balloon A a carbon-dioxide line is taken from the junction N to the filter at A to replace medium in flask B by carbon dioxide as the flask is emptied. This lead is clipped off close to A when the flask is to be changed, to obviate entry of air into the tubing. The culture vessel F has been replaced by one of similar design but of 250 ml. working volume, and the stirring gas flow increased to 250 ml./min. The rubber bung forming the top of the vessel is now as follows. A large diameter hole is bored centrally through the rubber bung and through this is inserted a combination glass-calomel electrode (E.I.L., type SHDN33. Electronic Instruments Ltd., Richmond, Surrey). This has a rubber sleeve part-way up it which, greased with silicone grease, makes the electrode a gas-tight fit

in the hole and allows the electrode to be inserted without damage to the glass tip. Arranged around this are the inlet and sampling tubes shown in the original diagram and also inlets for alkali and acid. These latter are stainless steel tubes of $\frac{1}{8}$ inch (0·32 cm.) outside diameter to the projecting tops of which are soldered steel tubes of larger diameter into which fit smaller tubes in the manner of a cone and socket joint. Also inserted through a further hole so that the silicone-greased ebonite cap is about $\frac{1}{2}$ inch (1·27 cm.) into the bung is an E.I.L. platinum electrode type EPT 23. The steel tubing joints on the acid and alkali inlets are connected to small bore glass tubes which pass via stopcocks to rubber tubing which can be opened or closed by a Pye titrator delivery unit (Pye Instruments, Cambridge) adjusted so that each solenoid opens to the same extent. The upper ends of these tubes are connected to glass joints and then by glass tubing to alkali and acid reservoirs at a height of about 18 inches (45·7 cm.) above the culture vessel. Sterile air is admitted to these reservoirs through cottonwool plugs and 'Carbosorb'-containing tubes. The platinum electrode circuit is a recent addition and was not used in all the experiments reported here, and may be omitted. The glass electrode is connected to an E.I.L. pH meter/controller model 91 B, and the platinum electrode to a similar instrument graduated in millivolts (this is at present used only as a meter). The calomel reference electrode can be connected by means of a switch to either the pH meter or the millivoltmeter in circuit with either the glass or platinum electrode. The pH meter is also connected to a 'Dwarf' recorder (Everett-Edgcumbe Ltd., Colindeep Lane, London), and via the upper and lower controller circuits to the solenoids actuating the inflow of acid or alkali. In use the lower indicator of the pH meter/controller is set at the appropriate pH value. In most cases control necessitates only the addition of alkali as fermentation tends to lower the pH value of the culture medium (from its initial value pH 6·8) and the upper indicator is set about 0·2 units higher to act mainly as a safety device. The taps on the alkali and acid lines are then set by trial so that a very small amount of alkali is added at each opening of the solenoid valve and overshoot of pH is negligible. When an Eh measurement is to be made the pH meter is returned to the check point (after moving the upper control setting if necessary), the reference electrode is switched to the millivoltmeter and this is then set to the 'read' position. A stable Eh reading is usually obtained in about 5 min. and during this time the pH of the culture does not alter appreciably. To resume pH control the procedure is then reversed. This circuit was adopted so that a separate reference electrode need not be inserted in the culture vessel, but if the Eh value were to be continually recorded then a second electrode would be needed. No 'poisoning' of the platinum electrode by culture constituents has been noted in runs lasting many hundreds of hours.

Originally a pH controller working by mechanical contacts on a 'slave' meter operated by a Pye pH meter was used in the circuit, but the contacts on this were liable to stick in the 'on' position and breakdowns were comparatively frequent. The E.I.L. meter/controller is much more compact and reliable, and extremely stable.

All the parts of the apparatus including the solenoid valve tubings and the acid and alkali reservoirs and the acid and alkali solutions are sterilized by autoclaving (120°, 25 min.) and assembled aseptically. The two electrodes are sterilized by immersion for 24 hr in a solution of 1% (v/v) H_2SO_4 in 70% (v/v) ethanol in water. This treatment has had no adverse effects on the electrodes, and no contamination of the cultures attributable to the electrodes has occurred. The pH meter is initially

standardized with a second electrode system and external buffers, the culture electrode is then connected up and the pH of the uninoculated medium determined. This can then be checked against the pH value of a sample of the medium measured on an external pH meter. The check point of the pH meter is noted at about weekly intervals during a run, but usually drift amounts to only about 0·1 pH units per fortnight.

In some chemostat pH control systems (e.g. Wright, 1960) a timer is used to add the acid or alkali at short intervals during the 'on' cycle. With the taps in the alkali/acid addition circuit as described here the rate of addition of the solutions (3 % NaOH, w/v, or H_2SO_4, v/v) can be adjusted so that overshoot is negligible and our pH control is to better than 0·1 unit. Some adjustment of the relative positions of the acid and alkali inlets and the glass electrode may be needed to get optimum control.

A difficulty of growing the anaerobic bacteria used in the present work is the diffusion of air through the rubber tubing used in the medium circuits. This is negligible with some rubbers, but with other rubber tubings we have found that although air diffusion may not oxidize the medium to the point where a trace of the pink resazurin colour is seen, it can still increase the Eh value sufficiently to stop the growth of some organisms. Since rubber tubings, even from the same supplier, seem to vary from batch to batch we have not found any that can be unequivocally recommended. Neoprene is the best we have so far used, but this may split on autoclaving. Hungate (1963) and Hungate, Smith & Clarke (1966) have also considered the problem of diffusion of oxygen through rubber in anaerobic cultures. The latter authors recommend butyl rubber stoppers for culture tubes. The wall thickness of the tubing should be as great as possible consistent with flexibility. Although much of the tubing may be of glass or stainless steel some rubber is needed, in parts, to give flexibility to take up strains and vibrations in the apparatus (and during autoclaving) and to allow joints to be connected.

Bacteria. The lipolytic Bacterium 5S was the strain previously used and described by Hobson (1965*a*). Two strains of *Bacteroides amylophilus* were isolated by our colleague Dr T. H. Blackburn and were similar to the strains of *B. amylophilus* described by Blackburn & Hobson (1962). This bacterium ferments only starch, dextrins or maltose and is one of the more important proteolytic bacteria in the rumen, producing amylase and protease, both excreted into the culture medium.

Media. The medium for growth of bacterium 5S on glycerol in an atmosphere of 100 % CO_2 was described by Hobson (1965*a*). For fructose growth the glycerol was replaced by fructose. When 95 % (v/v) N_2 + 5 % (v/v) CO_2 was used as atmosphere the sodium bicarbonate concentration in the medium was decreased to 0·02 % (w/v), and the cysteine hydrochloride solution brought to pH 6·6 with sodium hydroxide solution before the bicarbonate and carbohydrate were added and the solution filter-sterilized. In batch cultures under this atmosphere M/60 phosphate buffer (pH 6·6), was included to help to control the culture pH value. For incubations under an atmosphere of 100 % N_2 bicarbonate was omitted from this medium. The medium for batch and continuous culture growth of *Bacteroides amylophilus* (formulated by Dr T. H. Blackburn to give optimum growth in batch cultures) contained, per 100 ml.; mineral solutions *a* and *b* (Hobson, 1965*a*), 15 ml. each; Bacto tryptose, 0·1 g.; cysteine hydrochloride, 0·1 g.; sodium bicarbonate, 0·6 g.; resazurin, 0·1 ml. of 0·1 % solution; maltose hydrate (B.D.H.), to concentration required; water to 100 ml. Stock cultures were kept on slopes of this medium solidified with 2 % agar. This bacterium will grow

in a medium containing ammonia as sole nitrogen source, but the addition of tryptose to the medium appeared to decrease the lag phase in batch cultures, although no utilization could be shown (Blackburn, 1965).

General methods of preparing the media and of setting up and inoculating the continuous culture apparatus have been previously described (Hobson, 1965*a*), except that in the later work with *Bacteroides amylophilus* the maltose, bicarbonate and cysteine were added as concentrated filter- or heat-sterilized separate solutions and not as a mixed filter-sterilized solution.

Analytical methods. Methods of sampling the cultures and of determining dry weights of bacteria, glycerol, total volatile fatty acids (VFA), lactic acid and culture turbidity were as previously described (Hobson, 1965*a*). Succinic acid was determined by a manometric method (Umbreit, Burris & Stauffer, 1957). Fructose was determined by the method of Roe (1934) and maltose by the Somogyi–Nelson method (Nelson, 1944). Volatile fatty acids were separated by gas chromatography (on an instrument made by Gas Chromatography Ltd., Maidenhead) by our colleague Mr T. Walker. Bacterial-nitrogen was determined by a Kjeldahl method on bacteria centrifuged down from the culture at 0° and washed once with water at 0°. Total medium-nitrogen was determined by a Kjeldahl method and ammonia-nitrogen by a microdiffusion method. Lipase and esterase activities were determined by using naphthyllaurate or acetate as substrates at pH 6·8 (Hobson & Summers, 1966). Protease activity was determined by a modification of the method of Anson (1938) as used by Blackburn (1965), with casein as substrate. Amylase activity was determined by a modification of the method of Walker & Campbell (1963), again as used by Blackburn (1965). Total counts of Bacterium 5S were made on a Coulter Counter (Coulter Electronics Ltd., Dunstable, England) with a 30 μ orifice tube at an aperture current setting of 8 and a threshold value of 20 after suitable dilution of the culture in 0·9% (w/v) NaCl solution. *Bacteroides amylophilus* was counted at a threshold value of 35. In all cultures, batch and continuous, the culture turbidity was found to have a linear relationship to bacterial dry weight (mg./ml.) except at very low values of turbidity (about 3 turbidity units) and turbidity was used to monitor the continuous cultures. However, nearly all values of bacterial yields were calculated from actual dry weight measurements and not from turbidity readings.

Growth in the basal media, without carbohydrate, was so small that no accurate estimate of bacterial concentration either by weight or turbidity could be made and this degree of basal growth was neglected in calculating yields.

The definitions of bacterial yields, $Y_{\text{substrate}}$ and Y_{ATP}, are as proposed by Bauchop & Elsden (1960) and as used in the previous paper (Hobson, 1965*a*).

In all cases there were slight variations in substrate concentrations in the different lots of medium used during each continuous culture run and in different cultures. The nominal concentration of sugar is given, but values for Y and fermentation products are calculated on the analysis of the medium flowing through the culture at any particular time.

The rate of alkali addition to the cultures at pH values about 6–6·5 was small, about 5% of the medium flow rate; dilution rates are given as the actual medium flow rate.

All cultures were incubated at 39°.

RESULTS

Lipolytic Bacterium 5 S

Culture on glycerol. Batch cultures showed that glycerol-limited cultures could be run at a glycerol concentration of 59·7 μmole/ml. (Hobson, 1965*a*); Fig. 1 shows some results from a continuous culture of 1660 hr duration at this glycerol concentration under an atmosphere of CO_2. The pH value was controlled at pH 6·1 and, as in all the cultures described, at least 30 hr were allowed for the cultures to stabilize at a particular growth rate before measurements were begun. The optimum dilution rate (D) shown is about 0·2 hr^{-1}, but when the medium flow was held at *D* 0·19 hr^{-1} and the pH value decreased to 5·5 the culture rapidly washed out. At this dilution rate (0·19 hr^{-1}) at

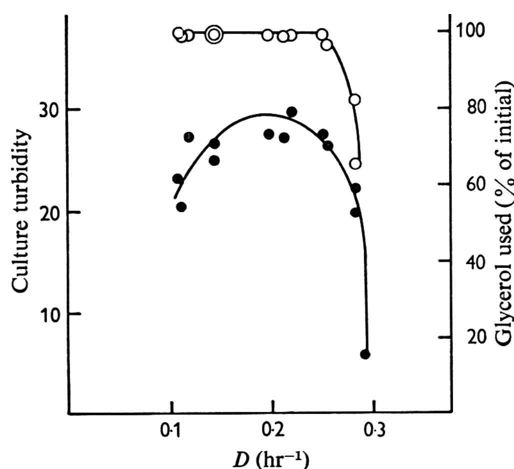

Fig. 1

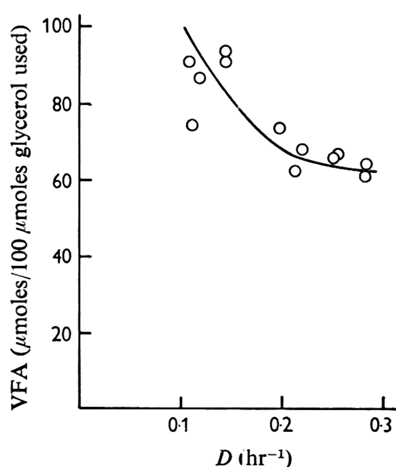

Fig. 2

Fig. 1. Culture of Bacterium 5S on glycerol. Culture turbidity ●. Glycerol used ○. Results from one culture of 1660 hr duration. A turbidity of 20 is equivalent to 0·79 mg. dry wt bacteria/ml. *D* in all figures = dilution rate.

Fig. 2. Culture of Bacterium 5S on glycerol. Volatile fatty acid (VFA) production. Results from culture shown in Fig. 1.

pH values above 7 the culture slowly washed out and at pH 7·4 when the dilution rate was changed to 0·21 hr^{-1} a rapid washout took place. Over the range pH 6 to 7 at *D* 0·2 hr^{-1} no differences in bacterial yield were found. Lipase and esterase activities were measured at different growth rates and two peaks of activity (coincident for lipase and esterase) in both bacteria and supernatant fluid were found at dilution rates of about 0·24 hr^{-1} and 0·1 hr^{-1}. The lipase activity of the bacteria showed a maximum at pH 6·6 when tested at four culture pH values. This organism produced acetic, propionic and succinic acids and a little lactic acid when fermenting glycerol (Hobson, 1965*a*); a change in the proportion of the volatile fatty acids with growth rate is shown in Fig. 2. There was a suggestion of a small increase in amounts of fatty acids formed as the culture pH changed from 6 to 7 at *D* 0·2 hr^{-1}.

Batch cultures with different concentrations of glycerol were incubated under atmospheres of oxygen-free CO_2, N_2+CO_2 (95+5) and N_2. At a glycerol concentra-

tion of 28·3 μmoles/ml. growth was equally rapid in all media and 98 % of the glycerol was utilized. However, at glycerol concentrations above this, lack of buffering capacity in the media under N_2+CO_2 or N_2 caused a decrease to about pH 5·6, when growth ceased before all the glycerol was utilized. In the medium under 100 % CO_2 glycerol up to 76 μmoles/ml. was utilized. The yield of bacteria per mole of glycerol fermented was similar in all cultures (about 17). These results showed that high concentrations of carbon dioxide were not essential for growth of the bacterium 5S, so continuous cultures were conducted with the medium as before but with the sodium bicarbonate concentration decreased to 0·02 % (w/v) and with a gas phase N_2+CO_2 (95+5). The results of two cultures of 300 and 400 hr duration are combined in Fig. 3. One culture

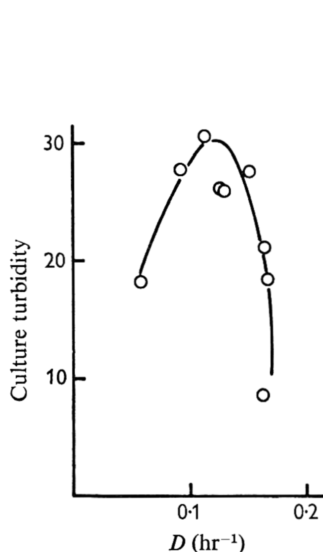

Fig. 3

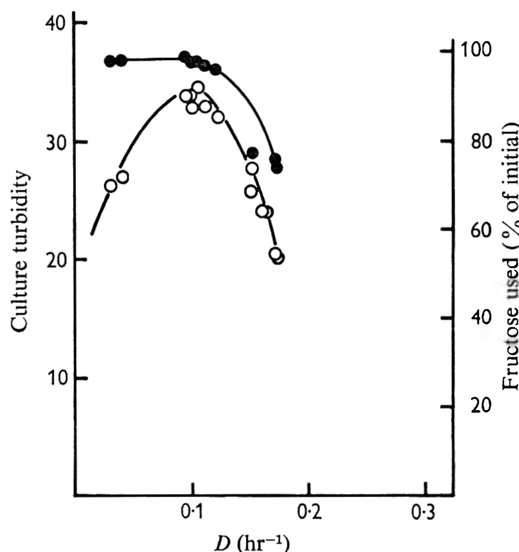

Fig. 4

Fig. 3. Culture of Bacterium 5S on glycerol under an atmosphere of N_2+CO_2 (95+5 by vol.). Results from two cultures of 300 hr and 400 hr duration. A turbidity of 20 is equivalent to 0·90 mg. dry wt bacteria/ml.

Fig. 4. Culture of Bacterium 5S on fructose. Culture turbidity ○. Fructose used ●. Results from a culture of 1150 hr duration. A turbidity of 20 is equivalent to 0·92 mg. dry wt bacteria/ml.

was controlled at pH 6·2 and the other at pH 6·5. When the culture was changed to pH 5·4 at D 0·12 hr^{-1} a rapid washout occurred. In the culture at pH 6·5 the values of $Y_{glycerol}$ were, at D 0·057 hr^{-1}, 15·8; D 0·092 hr^{-1}, 16·1; D 0·128 hr^{-1}, 20·2, and volatile fatty acids produced were over 90/100 μmoles glycerol used. Glycerol used was 97 % of that added at dilution rates about 0·1 hr^{-1}.

Culture on fructose. Batch cultures containing different concentrations of fructose showed that the growth of Bacterium 5S was proportional to fructose used at concentrations of fructose up to 30·6 μmoles/ml. Above this concentration pH changes stopped growth before all the fructose was utilized. The fermentation products were in all cases acetic and propionic acids at a total of 200 μmoles/100 μmoles fructose used and in the amounts 55 μmoles acetic acid and 145 μmoles propionic acid. Growth between 18 and

24 hr of incubation was logarithmic. A value for growth rate between these times was calculated by using the equation

$$\mu t = 2{\cdot}303\,(\log n - \log n_0),$$

where n and n_0 were determined from the graph of culture turbidity against total count (see below). With increasing initial concentration of fructose (nominally 0·1, 0·2, 0·4, 1·0, 2·0%, w/v) values for μ of 0·108, 0·123, 0·125, 0·167, 0·161 hr^{-1} were obtained.

Continuous cultures of Bacterium 5S were run at a fructose concentration of 27·8 μmoles/ml. The results from a culture of 1150 hr duration at pH 6·2 are shown in Fig. 4. Total counts were made on a number of samples from this culture; Fig. 5 shows that total bacterial numbers were in linear relationship to bacterial mass con-

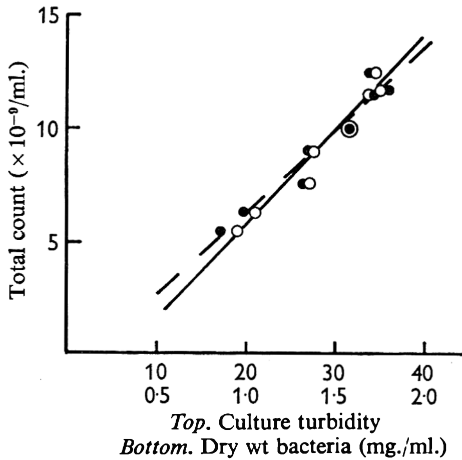

Fig. 5

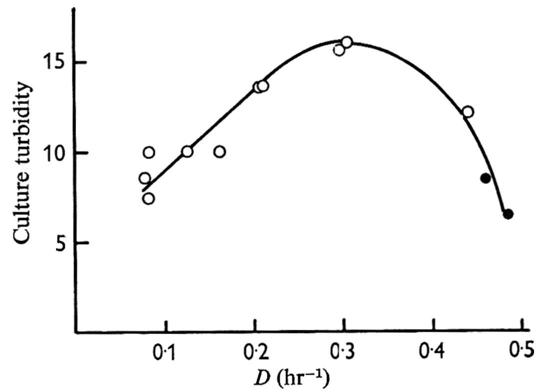

Fig. 6

Fig. 5. Culture of Bacterium 5S on fructose. Total count ($\times 10^{-9}$) = 0·42 turbidity −2·7. Culture turbidity ○. Total count ($\times 10^{-9}$) = 7·31 dry bacteria −1·0. Bacterial dry weight ●.

Fig. 6. Culture of *Bacteroides amylophilus* on maltose. Results from a culture of 890 hr duration. A turbidity of 14 is equivalent to 0·9 mg. dry wt bacteria/ml. At points indicated ● a true steady state was not obtained (see text).

centration and to culture turbidity. The bacterial yield (Y_{fructose}) increased to a maximum of 60 (average of four determinations) at dilution rates about 0·1 hr^{-1}. The fermentation products were acetic and propionic acids in total amounts about 200 μmoles/100 μmoles fructose used at all dilution rates. No difference was found in culture turbidity at culture pH values of 6·2 and 6·5 at D 0·08 hr^{-1}. At D 0·1 hr^{-1} changing the culture pH to 5·6 or 7·0 resulted in a slow washout of the cells.

Bacteroides amylophilus

Culture on maltose. Batch cultures of *Bacteroides amylophilus* at different maltose concentrations showed that growth was proportional to maltose used at concentrations up to 11·7 μmoles/ml. and above this concentration low pH values or lack of nitrogen limited the growth. The amount of maltose left at the end of the log phase of growth was similar in each culture and was about 0·82 μmoles/ml. When growth ceased lysis

of the organisms was rapid and the culture turbidity decreased. Acetic, formic and succinic acids were formed. The total volatile fatty acids formed during the log phase of growth at all concentrations of maltose were similar and averaged 138 μmoles/100 μmoles maltose used; succinic acid was not determined quantitatively. The yields of bacteria per mole of maltose used ($Y_{maltose}$) during the log phase of growth at different initial maltose concentrations from 2·04 to 11·7 μmoles/ml. were: 110, 102, 87, 78, 77, respectively. Since the culture turbidity was small for this bacterium, *B. amylophilus*, an accurate determination of total count from culture turbidity was not possible at low concentrations of maltose in the batch cultures, but at higher concentrations where the culture turbidity corresponding to n_0 was higher, values for total counts and hence growth rates could be determined. These suggested a maximum value of μ of about 0·46 hr^{-1}.

Continuous cultures were run at a maltose concentration of 7·6 μmoles/ml. Figure 6 shows results from a run of 890 hr at a culture at pH 6·4. When the dilution rate was set above 0·443 hr^{-1}, at D 0·460 hr^{-1} and 0·485 hr^{-1}, the culture turbidity decreased rapidly to 8·5 and 6·5 units, respectively; but at each dilution rate the rapid decrease was followed by a slow decline at the rate of about 1 unit in 15 hr, so a true steady state was not achieved. At the dilution rate 0·485 hr^{-1} the maltose remaining in the culture was 1·93 μmoles/ml. At the other dilution rates the average amount remaining was 0·58 μmoles/ml.; this corresponds to a maltose utilization of about 92%.

Total counts of bacteria were made during continuous culture of *Bacteroides amylophilus* at different culture turbidities and dilution rates. A linear relationship was found between turbidity and total count; a turbidity of 8 corresponded to a total count of $3{\cdot}4 \times 10^9$ bacteria/ml.

Figure 7 shows the values of Y (per mole of maltose utilized) obtained during the above culture and during another of 1100 hr duration. Figure 8 shows the volatile fatty acids formed at different dilution rates; these acids were identified as acetic and formic in equimolar amounts. The succinic acid, the only other fermentation product, formed was determined at two dilution rates. At D 0·296 hr^{-1} fermentation products, per 100 μmoles maltose used, were 172 μmoles formic acid, 172 μmoles acetic acid and 159 μmoles succinic acid. Assuming uptake of CO_2 in succinic acid formation this gives a carbon recovery of 82·6 %. At D 0·077 hr^{-1} the products were 126 μmoles formic acid, 126 μmoles acetic acid and 201 μmoles succinic acid, giving a carbon recovery of 81·7%.

The Eh value of the medium as measured by the platinum and calomel electrode system was about -100 mV. at pH 6·5 (an actual reading of about -350 mV.), and the resazurin was entirely colourless. At this Eh value growth of the bacteria took place, but this growth did not result in any decrease of the Eh value, and when the Eh value increased, for any reason, the growth of the bacteria ceased and they began to wash out. The bacteria appeared to have no capacity for reducing the medium even when a heavy concentration of bacteria was present, although a (presumptive) Bacillus contaminant rapidly decreased the Eh value, even when present only in small numbers.

The morphology of the *Bacteroides amylophilus* bacteria in batch and continuous cultures varied from small cocci to small rods, with sometimes some of the irregularly shaped bacteria first noted by Hamlin & Hungate (1956). The number of irregular-shaped organisms increased under adverse conditions, such as high Eh value or low

pH value at the end of batch cultures; but the coccoid or rod-like morphology appeared to be independent of the growth rate. The same relationship between culture turbidity and bacterial dry weight was found whether the bacteria were predominantly coccoid or rod-shaped. The bacteria were capsulated at all times, but at dilution rates round about 0·3 hr^{-1} the culture foamed more than at lower or higher rates, and this seemed to be associated with an increase in capsule and slime production, although no marked difference in the nitrogen content (about 9–10% of the dry weight) could be found in bacteria grown at dilution rates of 0·148, 0·222 and 0·354 hr^{-1}.

During two continuous cultures of *Bacteroides amylophilus* determinations were made of bacterial-nitrogen and total-nitrogen in culture supernatant fluid and inflowing medium and ammonia-nitrogen in culture supernatant fluid and medium. Six

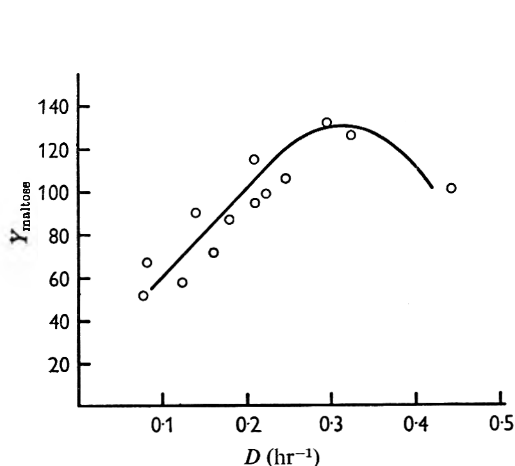

Fig. 7

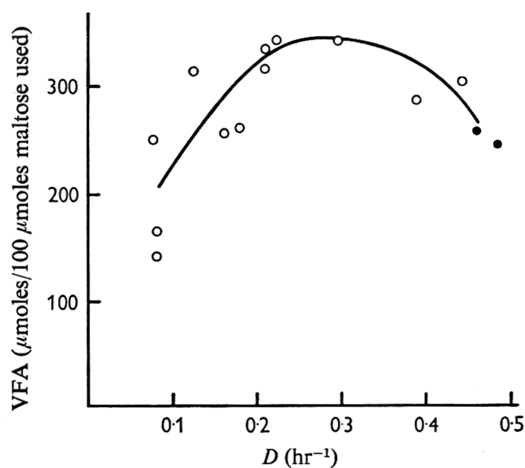

Fig. 8

Fig. 7. Culture of *Bacteroides amylophilus* on maltose. Results from two cultures of 890 hr and 1100 hr duration. $Y_{maltose}$ is calculated on the basis of maltose used, not just that fermented (see text).

Fig. 8. Culture of *Bacteroides amylophilus* on maltose. Volatile fatty acid (VFA) production. Results from the two cultures shown in Fig. 7.

determinations were made at dilution rates of 0·10 and 0·21 hr^{-1}. These showed that the ammonia disappearing from the medium was equivalent to the bacterial nitrogen formed (NH_3-N was an average of 104% of cell N), so that the bacterium was using ammonia in preference to the amino acids of the tryptose. This type of nitrogen utilization has been found with other rumen bacteria (see review by Hungate, Bryant & Mah, 1964). Although the ammonia in the medium was rather less than that calculated from the constituent salt concentrations it was still present in excess in the culture and so could not have been limiting the growth of the bacteria.

The original strain of *Bacteroides amylophilus* used in the cultures described above changed in stock cultures in that it became slower growing, and it was found impossible to run continuous cultures over the range of dilution rates previously used. Further cultures were then made with a second strain which had been classified by the usual tests as identical to the previous one. This second strain grew over a similar range of dilution rates and produced the same fermentation products and enzymes, but it

differed in the slope of the culture turbidity/dry weight relationship, giving a higher turbidity for the same dry weight. Also at dilution rates round about 0·3 hr^{-1} the cultures foamed more than did the previous ones and at the high culture turbidities found here some 30% of the bacterial mass was a slime excreted into the culture medium. This second strain was used to continue and confirm the observations on enzyme activities begun with the previous strain. In a run of 1000 hr the total amylase and protease activities per ml. of the culture were measured at seven dilution rates between 0·09 and 0·41 hr^{-1} and corrected to activity per unit weight of bacteria in the culture. Both the protease and amylase activities showed a peak at a dilution rate about 0·2 hr^{-1} and the amylase activity showed a second peak at 0·09 hr^{-1} or less. A culture was run at a dilution rate of $0{\cdot}283 \pm 0{\cdot}006$ hr^{-1} for 490 hr and the culture was adjusted to seven values between pH 5·1 and 6·8. At pH 6·8 the culture washed out and at pH 5·1 a very granular growth occurred with a variable culture turbidity. At all intermediate pH values the culture turbidity was the same. Amylase activity showed a maximum at a culture pH of 6·1. Protease activity increased rapidly between pH 6·6 and 6·4 and then showed a slight increase as the culture changed to pH 5·1. The fermentation products also changed, the amount of volatile fatty acids produced per mole of maltose utilized showing a maximum at pH 5·8. Succinic acid was not determined.

DISCUSSION

The results given here again show that it is possible to grow strictly anaerobic bacteria for prolonged periods in continuous culture, but the results of the Eh measurements indicate why care must be taken to eliminate even the slightest traces of oxygen from both continuous and batch cultures. The inability of *Bacteroides amylophilus* to reduce the medium agrees with some unpublished experiments where we found that suspensions of *Selenomas ruminantium*, Bacterium 5S, *Ruminococcus albus* and *B. amylophilus* showed no oxygen uptake in manometric experiments, whereas rumen anaerobes such as Veillonella species and *Peptostreptococcus elsdenii*, which can grow at higher culture Eh values than the former bacteria, showed a rapid oxygen uptake, as did the clostridia which were tested. Presumably bacteria such as these latter, which are found in comparatively small numbers in the rumen, together with some facultative anaerobes, are responsible for the oxygen uptake of rumen contents and the preservation of an Eh suitable for the predominant bacteria (typified by the former group) in spite of the air introduced into the rumen during feeding. In cases where volatile fatty acids (VFA) and lactic or succinic acids are the fermentation products the proportions of VFA to lactic or succinic acids alter with growth rate. The results obtained here with pH-controlled cultures support the suggestion made previously (Hobson, 1965*a*) that the pH range for optimum growth of bacterium 5S is small (about pH 5·7–7). *B. amylophilus* will grow at a lower pH than 5S or *S. ruminantium* (Hobson, 1965*a*) and although the rumen pH value of the conventionally fed animal does not usually go below about pH 5·7 the rumen pH of animals fed on high-starch rations in which *B. amylophilus* may be important often decreases to as low as 5. Again confirmed are the comparatively low growth rates of the anaerobic rumen bacteria; and the maximum growth rates in batch cultures compared well with those obtained in continuous cultures. In the case of Bacterium 5S the concentration of carbon dioxide in the culture atmosphere had a profound effect on growth rate, but not on yield of bacteria or

fermentation products. Why this should be so is not apparent. Growth of Bacterium 5S was also slower on fructose than on glycerol.

Hobson & Summers (1966) reported the presence of two peaks for lipase and esterase production at different growth rates for Bacterium 5S growing on fructose. The present results show that this also held for growth on glycerol. Enzyme production was also a maximum in a culture at pH 6·6, which was about the optimum pH value for growth. The results obtained with *Bacteroides amylophilus* indicate that the amylase and protease activities of this bacterium also vary with growth rate, and with culture pH value. The evidence of changes in pattern of fermentation products with growth rate presented here and in the previous paper (Hobson, 1965*a*) is indicative of change in production of the enzymes of the fermentative pathways. Tempest & Herbert (1965) observed variations in rates of synthesis of the 'constitutive' oxidative enzyme systems of *Torula utilis* at different growth rates. Dawson (1965) observed that the intracellular amino acid pool of *Candida utilis* varied with growth rate during C- and N-limited chemostat culture, and Wright & Lockhart (1965) observed variations with growth rate in the antigenic composition of *Escherichia coli* organisms. There is thus increasing evidence for changes in the enzymic as well as the structural make-up of bacteria with growth rate as well as during growth between cell divisions.

The curve obtained by plotting bacterial concentration against dilution rate is of similar shape in all cases and like that found in previous experiments (Hobson, 1965*a*). However, in the case of Bacterium 5S growing on glycerol under 100% CO_2 there was a rather more abrupt washout at the higher dilution rates than found previously. This may have been due to a better control of pH value in the present apparatus. At dilution rates above 0·3 hr^{-1}, in the previous experiments the pH value rose from 6·2 to 6·6 and as this latter value is nearer the optimum for growth it may have given steady states at rather higher dilution rates than those found here. The decrease in bacterial concentration and bacterial yield at low dilution rates suggests a maintenance requirement for energy at low growth rates. Curves for bacterial concentration and dilution rate similar in shape to those found for the rumen bacteria have been found by other workers with different organisms and substrates (e.g. Wase & Hough, 1966); perhaps this type of relationship is more common than that found for *Aerobacter cloacae* by Herbert, Elsworth & Telling (1956), for which they derived theoretical equations.

In the previous paper (Hobson, 1965*a*) the bacterial yield ($Y_{glucose}$) of *Selenomonas ruminantium* was shown to be about 62 g./mole glucose fermented at optimum growth rates, and Hungate (1963) reported a value of about 55 g. bacteria/mole hexose fermented for *Ruminococcus albus* growing at one dilution rate on cellobiose with ammonia as nitrogen source. The results of the present work show that Bacterium 5S has a $Y_{fructose}$ of 60 at optimum growth rate. *Selenomonas ruminantium* and Bacterium 5S need amino acids and were growing in a complex medium. *Bacteroides amylophilus* utilises ammonia as nitrogen source, and the fermentation balance suggests some 20% of the maltose was used as a source of bacterial carbon. The yield of *B. amylophilus* at optimum growth rate then becomes 160 g./mole maltose fermented (from Fig. 7). The fermentation pathways of these bacteria have not been investigated in detail, but assuming that 2 ATP are generated in the production of 2 pyruvate from hexose and that 1 ATP is formed in the conversion of pyruvate to acetate, propionate or succinate, we obtain values of Y_{ATP} of about 20 for *B. amylophilus* or *S. ruminantium*

and 15 for Bacterium 5S growing on fructose. These values are higher than 10, which is the universal value suggested initially by Bauchop & Elsden (1960) and since found for some other bacteria. On the other hand the value for $Y_{glycerol}$ for Bacterium 5S (growing on glycerol) found here and in the previous experiments is 20, which corresponds to a value for Y_{ATP} of 10, assuming that 2 ATP are formed in glycerol fermentation. These calculations also assume that the enzymes liberated into the culture medium constitute a negligible proportion of the protein synthesized by the bacterium. This would appear to be the case with *B. amylophilus* since the bacterial-nitrogen formed and ammonia-nitrogen utilized were similar. These results suggest that Y_{ATP} may not be a constant even at optimum growth rates for different bacteria, and with individual types of bacteria it will obviously vary with growth rate, if some energy is used for maintenance and not bacterial growth. This latter variation again emphasizes the point that the bacterial yields in batch cultures, which we found to be less in these experiments than under optimum continuous culture conditions, cannot generally be taken as a guide to maximum yield when a maintenance requirement is involved, since they are a 'synthesis' of bacterial yields at different growth rates during the culture cycle, few if any of which may be near optimum. The high yields of *B. amylophilus* and *R. albus* (Hungate, 1963) suggest that little ATP energy is needed for the synthesis of cell constituents from simpler compounds, in conformity with the results of Senez (1962).

The provision of a grant from The Wellcome Trust, for part of the equipment, is gratefully acknowledged. We are also indebted to our colleague Dr T. H. Blackburn for the strains of *Bacteroides amylophilus* used here.

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The Sensitivity of Pseudomonads to Ethylene-diaminetetra-acetic Acid

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SUMMARY

Forty-one strains representing 21 nomen species of the genus *Pseudomonas* and 2 of the genus *Alcaligenes* were screened for sensitivity to ethylene-diaminetetra-acetic acid (EDTA). Sensitivity of organisms was assessed by determining the release of intracellular solutes and the loss of viability under the action of EDTA. The latter criterion was found to be the more useful for the differentiation of sensitive and resistant organisms. Strains of *Pseudomonas diminuta*, *P. geniculata*, *P. iodinum*, *P. maltophilia*, *P. pavonacea* and possibly *P. rubescens* were considered as resistant to EDTA. Strains of *P. alcaligenes* were clearly differentiated from strains of *Alcaligenes faecalis*, which were highly resistant to EDTA. The practical value and possible taxonomic significance of the results are discussed.

INTRODUCTION

There is growing evidence that metal cations may play important roles in maintaining the structural integrity and permeability characteristics of the surface structures of a variety of Gram-negative bacteria (Vincent & Humphrey, 1963; Strange, 1964; Brown, 1964; MacLeod, 1965; Asbell & Eagon, 1966). The various effects of ethylene-diaminetetra-acetic acid (EDTA) on these organisms probably involve binding to or extraction of such cations. Thus, EDTA is known to potentiate the action of lysozyme (Repaske, 1956, 1958) and a variety of bactericides (MacGregor & Elliker, 1958; Gray & Wilkinson, 1965*a*; Leive, 1965*a*; Brown & Richards, 1965). It has also been found to increase the death rate of starved bacteria (Strange & Dark, 1965). It is generally considered that EDTA disorganizes an outer layer of the cell wall, facilitating penetration or action of the antibacterial agent. Recent work has confirmed that EDTA causes a relatively unspecific increase in the permeability of cells of sensitive organisms (Leive, 1965*b*; Hamilton-Miller, 1965), without necessarily killing or altering the growth rate of the cells (Leive, 1965*b*; Brown & Richards, 1965).

During his investigation of lysozyme potentiation, Repaske (1956, 1958) noted that EDTA alone had appreciable lytic activity against *Pseudomonas aeruginosa*, as judged by a decrease in the turbidity of suspensions of the organism. This observation, which was not made for other organisms tested, has been confirmed by other workers (Shively & Hartsell, 1964*a*, *b*; Eagon & Carson, 1965). It is now clear that EDTA can cause extensive damage to the cell wall of *P. aeruginosa* (Voss, 1964; Eagon & Carson, 1965), the release of intracellular solutes (Bernheim, 1963; Gray & Wilkinson, 1965*a*), and death of the organism (MacGregor & Elliker, 1958; Gray & Wilkinson, 1965*a*; Eagon & Carson, 1965). The extent of the lytic action of EDTA is very markedly

dependent on the experimental conditions used (Repaske, 1958; Wilkinson, 1962; Shively & Hartsell, 1964*a*, *b*). With isolated cell walls of *P. aeruginosa*, Gray & Wilkinson (1965*b*) found that EDTA solubilized material containing phosphorus and carbohydrate, and concluded that metal cations and lipopolysaccharide are essential components of these cell walls. Similar conclusions were reached by Eagon and his co-workers (Eagon & Carson, 1965; Carson & Eagon, 1966; Asbell & Eagon, 1966).

Of a range of organisms examined by Gray & Wilkinson (1965*a*), only three species of the genus *Achromobacter* (one of which was stated to be *Alcaligenes faecalis*) displayed the exceptional sensitivity to EDTA characteristic of *Pseudomonas aeruginosa*. However, these hypersensitive organisms may differ from other Gram-negative bacteria only in degree. Thus, lysis of organisms or solubilization of wall components by EDTA has been noted for species of the genus *Salmonella* (Colobert, 1957*a*, *b*; Herzberg & Green, 1964). Leive (1965*c*) reported the release of lipopolysaccharide from the walls of whole organisms of several strains of *Escherichia coli* treated with EDTA. This result was not obtained with the isolated cell walls of other strains (Edwards & Noller, 1964; Gray & Wilkinson, 1965*b*). Nevertheless, it seemed possible, as suggested by Shively & Hartsell (1964*a*), that sensitivity to EDTA might be an important character in the taxonomy of the pseudomonads.

This paper presents results obtained with a range of pseudomonads and some other organisms, during a general survey of Gram-negative bacteria for sensitivity to EDTA. Since the completion of the work, Wolin (1966) has reported a lytic action by EDTA on *Vibrio succinogenes*.

METHODS

In their survey of pseudomonads, Shively & Hartsell (1964*a*) measured the sensitivity of an organism to EDTA by the change produced in the turbidity of a suspension of the organisms. As such changes might result from simple osmotic effects (Bernheim, 1963) as well as from gross lysis, more direct methods of determining EDTA sensitivity were preferred in the present study. The general techniques used for measuring the bactericidal action of EDTA and the release of intracellular solutes from the bacteria were based on those used by Gray & Wilkinson (1965*a*).

Organisms. Most organisms were freshly obtained from the National Collection of Type Cultures (NCTC) or the National Collection of Industrial Bacteria (NCIB). A strain of *Pseudomonas aeruginosa* coded FB was obtained from Dr F. Bernheim, and the organism coded BR 1/2 from the Bacteriological Laboratory of Reckitt & Sons Ltd. (Dansom Lane, Hull). Individual strains are listed in Tables 1–3.

Cultural conditions. For the preparation of thick suspensions of bacteria, the organisms were grown for 24 hr on nutrient agar (Oxoid). Slopes were inoculated from test-tube cultures (24 hr) of the organisms in nutrient broth no. 2 (Oxoid). Strains of *Pseudomonas aeruginosa*, *P. alcaligenes*, *P. maltophilia*, *P. ovalis*, *P. iodinum*, *Alcaligenes faecalis*, *A. odorans* and an *Achromobacter* species were grown at 37°; all other organisms were grown at 25°.

Test procedure. Organisms were washed from the slopes with 0·2 M-borate buffer (pH 7·1) and the suspension was filtered through a glass sinter (no. 1 porosity). The bacteria were collected by centrifugation at 20°, washed twice, and finally dispersed as a thick suspension in the buffer. Bacterial dry weights were determined by drying samples (2 ml.) of suspension and of buffer overnight at 110°, and were within the range

4–18 mg./ml. Tests showed that variations in bacterial concentration within this range had only slight effect on the subsequent action of EDTA.

Samples (2 ml.) of bacterial suspensions were added to volumes (20 ml.) of each of the following solutions kept at 20°: (*a*) 0·0034 M-EDTA in the borate buffer; (*b*) borate buffer; (*c*) 0·0034 M-EDTA in borate buffer containing sucrose (0·5 M); (*d*) borate buffer containing sucrose (0·5 M). After 1 hr, samples (1 ml.) of suspensions from (*a*) and (*b*) were taken for viability determinations, then all suspensions were centrifuged (12,000 *g*, 5 min.). The supernatant fluids were decanted and clarified by further centrifugation (3,500 *g*, 30 min.). Supernatant fluids were similarly prepared from bacteria suspended in borate buffer as in (*b*), but heated for 10 min. at 100°. Repeat experiments, which gave satisfactory results, were done with most organisms. A similar series of tests (excluding sucrose-containing solutions) in which the borate buffer (pH 7·1) was replaced in all solutions and suspensions by 0·05 M-borate buffer (pH 9·2) was done with selected organisms.

Viability determinations. The number of viable organisms in a suspension was determined after serial dilution (usually 10^4–10^7 times) of the samples in 1% (w/v) aqueous NaCl. Volumes (0·5 ml.) were placed dropwise on plates of nutrient agar which had been dried for 2 hr at 60°. Colonies were counted after incubation for 24–48 hr at the original growth temperature for the organism.

Release of intracellular solutes. The extinction at 260 mμ of a supernatant fluid (corrected for test solutes) was used as a measure of the leakage of intracellular solutes during the test period. Extinctions were measured in 1 cm. silica cells in a Unicam SP. 500 spectrophotometer. Concentrations of solutes released were calculated as percentages of those from heated (100°) organisms.

RESULTS

The results of tests on 10 strains of *Pseudomonas aeruginosa* using EDTA at pH 7·1 (Table 1) confirmed the existing impression that sensitivity to EDTA is a characteristic property of the organism. In spite of the heterogeneity of bacteria grown in surface culture and the desensitizing effect of the borate buffer (Gray & Wilkinson, 1965*a*), EDTA consistently produced a leakage of material absorbing at 260 mμ and a substantial decrease in viability. Although the leakage of such solutes was usually about 30% of that from heated bacteria, from 2 strains it was nearly 60%. As both types of result have previously been obtained in similar tests with strain NCTC 1999 (Gray & Wilkinson, 1965*a*), it is doubtful whether strains giving the greater leakage are intrinsically more sensitive to EDTA. Also, because of rather high leakages into buffer alone in some instances, the increase in leakage produced by EDTA could not be relied upon as the sole index of sensitivity, as seemed possible from the earlier more limited study. For this reason, the decrease in viability caused by EDTA was introduced into the screening procedure as a second index. By using the latter index, the most sensitive strain of *P. aeruginosa* was strain FB (Bernheim, 1963), which was also the only one which did not produce either a visible or a fluorescent pigment.

In the presence of sucrose (0·5 M), the leakage induced by EDTA was substantially decreased (40–87%). Protection of the bacteria by sucrose, even when incomplete, could be taken as evidence that the action of EDTA was against the cell wall rather than the protoplast membrane. The ability of non-penetrating solutes, including NaCl

and sucrose, to inhibit lysis of pseudomonads treated with EDTA and lysozyme has previously been shown to depend on the species (Shively & Hartsell, 1964*b*).

In addition to *Pseudomonas aeruginosa*, it has been reported that *Alcaligenes faecalis* is also hypersensitive to EDTA (Gray & Wilkinson, 1965*a, b*). However, tests on 4 strains of this organism and 2 strains of *A. odorans* (Table 2) showed them to be highly resistant to EDTA. The resistance was maintained in similar tests at pH 9·2.

Table 1. *Sensitivity to EDTA of Pseudomonas aeruginosa*

Organisms were treated for 1 hr at 20° with 0·0031 M-EDTA in borate buffer pH 7·1 (T), buffer alone (B), and similar solutions containing 0·5 M-sucrose (T/S and B/S).

Strain	Suspension: Release of solutes absorbing at 260 mμ* T	B	T/S	B/S	Decrease in viability (%)†
NCTC 1999	26·4	2·5	8·6	2·2	75
NCTC 6750	37·1	2·9	20·7	10·4	95
NCTC 7244	32·0	7·2	21·1	12·5	99+
NCTC 8060	56·2	6·3	15·9	6·7	84
NCTC 8203	20·7	4·1	8·6	4·4	60
NCTC 8505	57·3	13·6	13·9	6·8	86
NCTC 10332	36·0	3·3	12·8	5·1	74
NCIB 8626	33·6	4·8	21·8	5·7	89
FB	22·3	1·8	5·1	2·2	99+
NCTC 5083 var. *erythrogenes*	42·8	12·3	29·9	11·5	93

* Expressed as a percentage of solutes from organisms heated 10 min. at 100°.
† Organisms in T, relative to those in B.

Table 2. *Sensitivity to EDTA of Alcaligenes species*

Organisms were treated for 1 hr at 20° with 0·0031 M-EDTA in borate buffer pH 7·1 (T), buffer alone (B), and similar solutions containing 0·5 M-sucrose (T/S and B/S).

Organism	Strain	Suspension: Release of solutes absorbing at 260 mμ* T	B	T/S	B/S	Decrease in viability (%)†
A. faecalis	NCTC 415	3·5	2·0	3·4	3·1	0
A. faecalis	NCTC 655	3·1	1·0	2·4	3·0	0
A. faecalis	NCTC 8764	8·3	6·6	7·3	6·6	0
A. faecalis	NCIB 8156	1·7	0·9	1·9	1·7	0
A. odorans	NCTC 10416	1·6	0·3	1·3	1·6	0
A. odorans var. *viridans*	NCTC 10388	2·5	0·8	3·1	3·5	0

* Expressed as a percentage of solutes from organisms heated 10 min. at 100°.
† Organisms in T, relative to those in B.

The original strain, BR 1/2, had been identified (1960) as *A. faecalis* on the basis of its cultural and physiological properties. In apparent confirmation, tests on *A. faecalis* NCTC 8769 showed that this organism also was highly sensitive to EDTA. However, strain NCTC 8769 was reclassified in 1964 as *Pseudomonas alcaligenes*, after Ikari &

Hugh (1963). Organisms of strain BR 1/2 have now been examined under the electron microscope; this showed that their flagellation was polar and monotrichous. Hence, this organism also should probably be renamed *P. alcaligenes* and is so described in Table 3. Tests on two other strains of *P. alcaligenes* (Table 3) confirmed the high sensitivity of this organism to EDTA, although it should be noted that strains NCTC 8769 and NCIB 9390 have recently been named as members of the new species *P. pseudoalcaligenes* (Stanier, Palleroni & Doudoroff, 1966).

Table 3. *Sensitivity to EDTA of Pseudomonas species*

Organisms were treated for 1 hr at 20° with 0·0031 M-EDTA in borate buffer pH 7·1 (T), buffer alone (B), and similar solutions containing 0·5 M-sucrose (T/S and B/S).

		Suspension				
		T	B	T/S	B/S	
Organism	Strain	Release of solutes absorbing at 260 mμ*				Decrease in viability (%)†
P. alcaligenes	NCTC 8769	20·7	0·9	8·2	2·5	98
P. alcaligenes	NCTC 10367	67·6	10·1	13·8	9·8	98
P. alcaligenes	NCIB 9390	73·3	10·4	12·8	8·4	99+
P. alcaligenes	BR 1/2	17·3	0·9	5·2	1·8	99+
P. aureofaciens	NCIB 9030	28·3	3·5	4·5	3·8	57
P. chlororaphis	NCTC 7357	26·6	1·5	3·8	1·8	42
P. denitrificans	NCIB 8376	11·5	1·3	4·5	4·0	52
P. diminuta	NCTC 8545	7·4	1·4	4·8	3·9	12
P. fluorescens	NCTC 10038	23·5	1·5	5·1	1·7	0
P. fluorescens	NCIB 9494	14·7	6·0	2·1	3·6	30
P. fragi	NCIB 8542	26·3	2·5	4·4	2·9	46
P. geniculata	NCIB 9428	32·8	5·5	14·2	3·4	0
P. graveolens	NCTC 8067	29·0	5·9	13·7	5·1	91
P. iodinum	NCTC 9742	5·9	1·9	5·6	7·4	0
P. maltophilia	NCTC 10257	18·6	5·9	7·8	6·3	11
P. maltophilia	NCTC 10259	12·7	4·2	7·6	5·4	0
P. maltophilia	NCIB 9201	31·2	1·9	5·3	2·7	0
P. mucidolens	NCTC 8068	23·4	7·5	14·0	7·1	42
P. ovalis	NCTC 912	38·9	9·7	23·0	10·4	85
P. pavonacea	NCIB 9395	13·3	3·1	11·8	7·0	35
P. putida	NCIB 9034	25·9	1·2	9·7	2·6	23
P. rubescens	NCIB 8768	14·2	1·3	4·9	3·2	0
P. stutzeri	NCIB 9040	32·9	5·0	7·1	3·4	99+
P. syncyanea	NCTC 9943	29·4	5·9	11·8	8·2	99+
P. synxantha	NCIB 8178	20·3	3·0	6·8	3·9	53
P. taetrolens	NCIB 9396	41·3	8·4	21·8	8·0	95

* Expressed as a percentage of solutes from organisms heated 10 min. at 100°.
† Organisms in T, relative to those in B.

Table 3 also records the results of tests at pH 7·1 on 18 other Pseudomonas species; except in two cases, only single strains were tested. The results for *Pseudomonas graveolens* and *P. taetrolens*, which are different cultures of the same strain, illustrate the degree of reproducibility obtained under such circumstances. Much closer agreement was generally obtained between the results of tests on different batches of organisms grown from the same stock culture. Of the 18 species, the strains of the following exhibited sensitivity to EDTA comparable with that of *P. aeruginosa*: *P. ovalis, P. stutzeri, P. syncyanea, P. taetrolens.* In fact, the bactericidal activity of

EDTA against *P. stutzeri* was greater than that against any other organism so far screened. Most of the remaining organisms displayed sensitivity to EDTA on one or both of the criteria used. However, as increased permeability of cells treated with EDTA is not restricted to pseudomonads, a more clear-cut differentiation of sensitive and resistant organisms in terms of a bactericidal action by EDTA was desirable. For this reason selected organisms were re-tested at pH 9·2 under conditions known to increase the sensitivity of *P. aeruginosa* NCTC 1999. The results of these tests are given in Table 4. The strains of *P. aureofaciens*, *P. chlororaphis*, *P. denitrificans* and strain

Table 4. *Effect of increased pH value on sensitivity to EDTA of Pseudomonas species*

Organisms were treated for 1 hr at 20° with 0·0031 M-EDTA in borate buffer pH 9·2 (T) and buffer alone (B).

Organism	Strain	Suspension T	Suspension B	Decrease in viability (%)†
		Release of solutes absorbing at 260 mμ*		
P. aeruginosa	NCTC 1999	26·9	4·0	99+
P. aureofaciens	NCIB 9030	18·1	3·7	99+
P. chlororaphis	NCTC 7357	18·1	3·9	99+
P. denitrificans	NCIB 8376	35·0	6·1	99+
P. diminuta	NCTC 8545	2·8	2·7	23
P. fluorescens	NCTC 10038	14·0	3·8	13
P. fluorescens	NCIB 9494	15·1	4·2	99
P. fragi	NCIB 8542	15·3	4·1	77
P. geniculata	NCIB 9428	11·5	11·4	0
P. iodinum	NCTC 9742	5·2	5·1	0
P. maltophilia	NCTC 10257	13·2	15·1	9
P. maltophilia	NCTC 10259	18·8	18·1	0
P. maltophilia	NCIB 9201	17·6	12·8	0
P. mucidolens	NCTC 8068	27·4	10·5	88
P. ovalis	NCTC 912	21·8	7·8	99+
P. pavonacea	NCIB 9395	9·6	9·3	29
P. putida	NCIB 9034	27·4	11·8	82
P. rubescens	NCIB 8768	20·7	8·6	63
P. synxantha	NCIB 8178	31·1	7·4	82

* Expressed as a percentage of solutes from organisms heated 10 min. at 100°.
† Organisms in T, relative to those in B.

NCIB 9494 of *P. fluorescens* were then clearly seen as sensitive organisms, while the strains of *P. fragi*, *P. mucidolens*, *P. putida*, *P. synxantha* and perhaps *P. rubescens* seemed to be rather less sensitive. Since *P. fluorescens* NCIB 9494 has recently been assigned to *P. putida* biotype A (Stanier *et al.* 1966), the differing results for the 2 strains of *P. fluorescens* are less surprising. With the remaining organisms (*P. diminuta*, *P. geniculata*, *P. iodinum*, *P. maltophilia*, *P. pavonacea* EDTA caused little or no leakage of solutes absorbing at 260 mμ, even with those strains which gave appreciable leakage at pH 7·1. These results might indicate that leakage from organisms swollen at the alkaline pH value (Shively & Hartsell, 1964*b*) masked any specific effect of EDTA on these organisms. The resistance of these organisms to EDTA is confirmed

by the fact that little or no decrease in viability was produced at either pH value (decreases in viability below 15% are considered to be of doubtful validity). With none of these resistant organisms was there evidence for any significant loss of viability in the pH 9·2 buffer alone.

DISCUSSION

It has been shown that sensitivity to EDTA is not confined to *Pseudomonas aeruginosa*, but occurs widely among both fluorescent and non-fluorescent pseudomonads and is found in representative organisms from at least 10 of the species centres proposed by Lysenko (1961). Although a proper assessment of the taxonomic value of this character must await more extensive tests on these and other Gram-negative organisms, several interesting and potentially useful correlations are already apparent.

A particularly striking example is the contrast in EDTA-sensitivity between *Pseudomonas alcaligenes* (polar, monotrichous flagellate) and *Alcaligenes faecalis* (peritrichous flagellate). These organisms have closely similar physiological properties and are normally differentiated only by the type of flagellation. High resistance to EDTA was also found for *A. odorans*, an organism which was removed from the genus *Pseudomonas* and placed in the genus *Alcaligenes* largely on the basis of its peritrichous flagellation (Målek, Radochová & Lysenko, 1963). Two Achromobacter strains (NCIB 8250 and an uncharacterized strain) proved to be similarly resistant to EDTA, suggesting that this property might help further in the recognition and classification of organisms in the genus *Pseudomonas* and the rather ill-defined genera *Alcaligenes* and *Achromobacter*.

Because of the lack of physiological standardization of the organisms used, the various pseudomonads tested probably cannot be arranged in a meaningful order of sensitivity to EDTA. However, *Pseudomonas aeruginosa*, *P. alcaligenes*, *P. stutzeri* and *P. syncyanea* may perhaps be differentiated from other sensitive organisms. EDTA had a potent lethal action on these organisms at pH 7·1, and the lysed organisms were centrifuged from suspension as a mucoid deposit which did not disperse on agitation. This effect was absent or much less obvious with other organisms, even when the bactericidal effect of EDTA was enhanced in tests at pH 9·2. These particularly sensitive organisms can be clearly differentiated from each other on the basis of phenotypic features (Stanier *et al.* 1966), and do not emerge as a distinctive group from Adansonian analyses nor from studies on DNA base composition (Colwell, Citarella & Ryman, 1965; De Ley, Park, Tijtgat & Van Ermengem, 1966; Mandel, 1966). However, the structure or composition of the cell wall has not been included as a character in any study of pseudomonads so far made. Although the extent of flagellation is of uncertain value in the internal differentiation of the pseudomonads (Rhodes, 1965), it is tempting to see it here as a unifying feature of *P. aeruginosa*, *P. alcaligenes*, *P. pseudoalcaligenes* and *P. stutzeri*; of the aerobic pseudomonads studied by Stanier *et al.* (1966) only these organisms were consistently monotrichous.

The position of *Pseudomonas syncyanea* is more confused. The strain used here (NCTC 9943 = ATCC 9979) has been successively labelled *P. syncyanea*, *P. mildenbergii* and *P. convexa* (Haynes, 1961), while Stanier *et al.* (1966) propose that *P. convexa* be reduced to synonomy with *P. putida*. According to *Bergey's Manual* (1957), *P. convexa* possesses 'a polar flagellum' but electron microscopic examination of

strain 9943 showed that the organisms had multitrichous flagellation. Preliminary experiments indicate that this organism is also exceptional in being lysed by pH 9·2 buffer alone.

The organism NCTC 10038 differed from all others tested in that whereas it could be considered sensitive to EDTA on the basis of leakage produced at pH 7·1 and 9·2, and also from turbidimetric studies and the potentiation of lysozyme (Shively & Hartsell, 1964*a*), EDTA had little or no lethal action on the organisms under the conditions of the present experiments. The only two other organisms accounted by Stanier *et al.* (1966) as biotypes of *Pseudomonas fluorescens* (*P. aureofaciens*, *P. chlororaphis*) included in the present tests gave virtually identical results (Tables 3, 4). These two organisms differed significantly from the remaining biotypes in DNA base composition (Mandel, 1966). As strain NCTC 10038 has been proposed as a neotype for *P. fluorescens* (Rhodes, 1959; Stanier *et al.* 1966), the possible significance of these observations calls for further work on biotypes of this species.

It would be premature to suggest that sensitivity to EDTA should characterize all organisms qualifying for the genus *Pseudomonas*. Nevertheless, it must be noted that the inclusion in the genus of each of the organisms found here to be resistant has been questioned by other workers. From studies on DNA homology and base composition, De Ley *et al.* (1966) concluded that *P. pavonacea* and *P. rubescens* should be removed from *Pseudomonas*, while the inclusion in the genus of *P. diminuta* and *P. iodinum* was considered doubtful. The generic status of *P. diminuta* was queried by Rhodes (1958) and Thornley (1960), while *P. iodinum* is exceptional in being non-motile, oxidase-negative and Gram-variable or Gram-positive. This organism is included in the current (1964) NCIB catalogue as a coryneform organism. Although EDTA at pH 9·2 consistently caused some decrease in viable counts of *P. diminuta*, *P. pavonacea* and *P. rubescens*, the absence of EDTA-induced leakage from the first two organisms indicates that the action of EDTA on them was different from that on more sensitive organisms. The growth of *P. rubescens* on the agar slopes was very heavy and the organisms were deposited from suspension only by centrifugation at high speed: the deposit appeared to consist of two layers, suggesting that a high proportion of degenerating organisms may have been present. The strain of *P. geniculata* tested was that used by Lysenko (1961) and De Ley *et al.* (1966) and therefore does not fit the description given in *Bergey's Manual* (1957). The heterogeneity of organisms labelled *P. geniculata* was noted by Mandel (1966) who reported that some of them have been identified as *P. maltophilia*. The present strain closely resembled *P. maltophilia* NCIB 9201 in its resistance to EDTA, in its weak or negative oxidase reaction and in the type of growth on nutrient agar. *P. maltophilia* can be distinguished from the main body of pseudomonads by the oxidase test and by its production of lysine decarboxylase (De Ley, 1964). In its phenotypic features the organism is very distinctive (Stanier *et al.* 1966).

It is a danger in a simple screening procedure such as that followed here that the somewhat arbitrary cultural and test conditions used might give rise to misleading results. To verify and explore the conclusions and correlations suggested by the results of these tests, a comparative survey of the composition of the cell walls of the organisms involved is being carried out. Like the Gram reaction, sensitivity to EDTA appears to depend on the structure of the cell wall so that this property might have a similar, if less general, taxonomic value.

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The Mechanism of Action of Proline Suppressors in *Aspergillus nidulans*

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SUMMARY

Effects of mutations in three independent proline suppressor loci were studied. Mutants of the *su-6* locus were almost completely deficient in ornithine transcarbamylase activity and mutants of the *su-19* locus produced ornithine δ-transaminase constitutively or semiconstitutively; this latter enzyme was strongly induced by arginine in the wild type. The *su-6* and *su-19* suppressor mutations were all recessive. Dominant and recessive mutants of the third suppressor locus, *su-2*, showed higher activities of arginase and ornithine δ-transaminase than did the wild type. It is suggested that in all three cases the enzyme alterations caused by the suppressor mutations allow the synthesis of proline by an alternative route, replacing the blocked major pathway.

INTRODUCTION

One of the mechanisms proposed by Wagner & Mitchell (1964) for explaining the mode of action of suppressor genes was that they open up alternative pathways, bypassing the metabolic block caused by the suppressed mutation. In fact, most suppressors studied have been found to act in different ways, and the only case known where the above explanation can be applied is that of the suppressors of acetate mutants in *Neurospora crassa* (Strauss & Pierog, 1954). However, there are several cases known where the biochemical situation leads one to suspect the possibility of the occurrence of this mechanism of suppression. One of them is the synthesis of proline.

The results of intensive investigations carried out on various microorganisms (Davis, 1955; Vogel, 1955; Strecker, 1957; Vogel & Kopac, 1959; Middelhoven, 1963; Vogel & Vogel, 1963; Middelhoven, 1964) showed that proline is synthesized from glutamate through glutamic-γ-semialdehyde (GSA) and Δ^1-pyrroline-5-carboxylic acid. This is a major route of proline synthesis, but proline can also be formed from ornithine via GSA (Fig. 2). This alternative route operates in the presence of an excess of exogenously supplied ornithine or arginine when, as was found by Andersson-Kotto & Ehrensvard (1963) in the wild strain of *Neurospora crassa*, the major route is almost completely replaced by the alternative one.

In *Aspergillus nidulans* two distinct loci, *pro-1* and *pro-3*, are known (Forbes, 1956), mutants of both loci responding to proline, ornithine, citrulline or arginine. According to the biochemical data they should be considered as blocked between glutamate and GSA. The occurrence of mutants of this kind, known also in other micro-organisms,

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proves that the endogenously produced ornithine or arginine is not sufficient to satisfy the proline requirements of the organism when the major proline route is blocked. In this particular situation one can expect that some mutations affecting the pathway of arginine synthesis and causing, for example, the accumulation of arginine, should act as proline suppressors.

The aim of the experiments presented in this paper was to find whether the above consideration is true for proline suppressors in *Aspergillus nidulans*. The genetical analysis of these suppressors involving mapping and studies on complementation was done previously (Weglenski, 1966). It was established that mutants of the three suppressor loci so far mapped are non-specific, i.e. they suppress mutants of *pro-1* and *pro-3* loci. The idea that their action involves alternative metabolic pathways is thus quite plausible. All mutants in two of the suppressor loci (*su-6* and *su-19*) are recessive, but in the third (*su-2*) both dominant (symbol *SU*) and recessive (*su*) mutations were mapped. Other symbols used are: *pro* = proline; *pab* = *p*-aminobenzoic acid; *ade* = adenine; *phe* = phenylalanine; *bio* = biotin.

METHODS

Material. All suppressor mutants of *Aspergillus nidulans* used in this work were obtained as spontaneous mutants from the *pro-6 pab-9 bio-1* strain; *pro-6* is an allele of the *pro-1* mutant and was originally obtained from the Department of Genetics, Glasgow University. The strains used for the enzyme assays were of the $pro^+ su^+$, $pro\, su^+$, *pro su* and $pro^+ su$ types, all marked additionally with the *ade-9*, *y* and *phe-2* mutations.

Media. Liquid minimal medium for culture of mycelium was the same as described by Cove (1966) with addition of $NaNO_3$ 6 g./l. medium. The final concentration of an amino acid when present in the medium was 0·002 M. All other media used were as described by Pontecorvo *et al.* (1953).

Culture and harvesting of mycelium. Mycelium was grown in one litre or 250-ml. Erlenmayer flasks containing 200 or 100 ml. medium, respectively. Flasks were inoculated with a heavy conidial suspension in water and were incubated at 30° for 35–40 hr (unless indicated otherwise) on a rotary shaker operating at 160 rev./min. After incubation the medium was filtered off and the mycelium rinsed with distilled water. Blotted mycelial pads were stored at −20°.

Enzyme extraction. Frozen mycelium mixed with glass powder was ground by hand in a chilled mortar with about 6 vol. of appropriate buffer. The resulting slurry was then centrifuged at 14,000*g* for 20 min at 4° and the supernatant fluid used for enzyme assays.

Assays of ornithine transcarbamylase (*OTC*). The reaction mixture for OTC contained 10 μmole ornithine HCl, 10 μmole carbamoyl phosphate, 100 μmole tris HCl (pH 8·3) and 0·3 ml. enzyme extract (equiv. 0·6 mg protein) in 0·1 M-sodium phosphate buffer (pH 8·0); total volume of reaction mixture 1·0 ml. After incubation at 35° for 15 min. the reaction was stopped by adding 2 ml. 0·5 M-perchloric acid solution and the precipitate removed by centrifugation. Citrulline was estimated colorimetrically by the method of Archibald (1944) by using an EEL colorimeter, blue filter no. 602.

Assay of ornithine δ-transaminase (*OTA*). The reaction mixture for OTA contained 10 μmole ornithine HCl, 10 μmole α-ketoglutarate, 50 μmole potassium phosphate (pH 8·0), 2 μmole pyridoxal -5- phosphate, 0·2 ml. enzyme extract (equiv. 0·4 mg.

protein) in a total volume 0·5 ml. Enzyme extracts were prepared in 0·1 M-potassium phosphate buffer (pH 8·0). The mixture was incubated at 37° for 10 min. and the formation of GSA was shown with *o*-aminobenzaldehyde and measured according to the method of Albrecht, Scher & Vogel (1962).

Assay of arginase. The reaction mixture for arginase contained 10 μmole arginine HCl (pH 9·5) and 0·2 ml. enzyme extract (equiv. 0·4 protein) in total volume 0·7 ml. Enzyme extracts were prepared in 0·002 M-maleic acid (pH 7·0) containing 1 μmole $MnCl_2$/ml., and were dialysed for 3 hr against three changes of the same solution. Incubation was for 10 min. at 37°, and the reaction was stopped by adding 0·3 ml. 6 N-HCl. The resulting protein precipitate was removed by centrifugation and the appropriately diluted supernatant fluid used for the determination of ornithine by the method of Chinard (1952).

Determination of protein. Protein estimations were made according to the Lowry *et al.* (1951) method, with crystalline bovine serum albumin as a standard.

RESULTS

Effect of suppressor mutations on ornithine transcarbamylase (OTC) activity

Strains carrying the *su-6* mutation show very poor growth on minimal medium. The growth of these strains can be stimulated by arginine (Weglenski, 1966). This suggested that they are deficient in one of the enzymes in the arginine pathway. It was found that the *su-6* mutation affects the activity of OTC, the enzyme which converts ornithine to citrulline. The specific activities of OTC in the *su-6* mutants are shown, together with those of the wild strain and of the other suppressor strains, in Table 1.

Table 1. *Aspergillus nidulans: specific activities of ornithine transcarbamylase (OTC) in wild-type and suppressor strains*

Suppressor locus	Genotype of strain	OTC activity (μmoles citrulline/mg. protein/hr)
.	*pro⁺ su⁺*	44·0
su-6	*pro-6 su-6*	1·5
	pro-6 su-16	1·8
	pro⁺ su-6	0·7
	pro⁺ su-16	0·9
su-19	*pro-6 su-19*	59·0
	pro-6 su-21	34·0
	pro-6 su-25	40·0
su-2	*pro-6 su-2*	59·0
	pro-6 su-17	42·0
	pro-6 SU-1	45·0
	pro-6 SU-8	43·0

Effects of suppressor mutations on ornithine δ-transaminase (OTA) activity

The activities of OTA in various strains are given in Table 2. This enzyme was inducible by arginine in all strains, though the induction in the strains carrying a mutation of the *su-2* locus seemed to be somewhat less effective. Strains carrying a mutation of the *su-19* locus showed a very high OTA activity when grown on minimal medium; in one case (*pro-6 su-25*) the activity was equal to the fully-induced value of

Table 2. *Aspergillus nidulans specific activities of ornithine δ-transaminase (OTA) in pro⁺ su⁺, pro su⁺, pro su and pro⁺ su strains grown on minimal medium (MM) and media supplemented as indicated*

Suppressor locus	Genotype of strain	MM	+proline	+ornithine	+arginine
		Media			
		OTA activity (mμmoles GSA/mg. protein/min.)			
.	*pro⁺ su⁺*	20·6	6·1	13·5	280·0
.	*pro-6 su⁺*	.	47·6	14·9	312·0
su-6	*pro-6 su-6*	38·2	35·1	15·2	264·0
	pro-6 su-16	35·1	29·2	14·6	289·0
	pro⁺ su-6	15·1	10·1	14·6	290·2
su-19	*pro-6 su-19*	143·0	140·8	83·4	300·0
	pro-6 su-21	146·0	152·5	112·8	287·0
	pro-6 su-25	295·2	295·4	216·0	295·0
su-2	*pro-6 su-2*	47·7	32·3	16·1	185·6
	pro-6 su-17	48·0	19·2	14·1	206·0
	pro-6 SU-1	50·1	34·0	22·4	212·0
	pro-6 SU-8	43·9	36·5	28·0	205·5
	pro⁺ su-2	34·4	28·1	32·6	167·1
	pro⁺ SU-1	48·2	66·1	38·1	195·0

the enzyme. Mutations in the *su-2* locus caused an approximately twofold increase of the OTA activity in comparison with that in the wild strain, whether a dominant or a recessive suppressor mutation was present. OTA was repressed by proline in the wild strain. There was no repression by proline of this enzyme in *su-19* strains or in *su-2* strains of the *pro⁺ su* type. However, in the strains of *pro su* type carrying the mutation

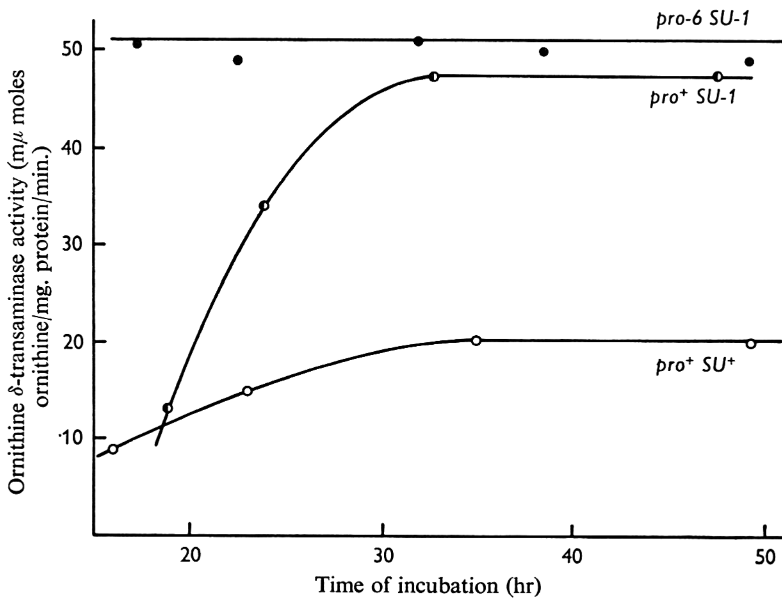

Fig. 1. *Aspergillus nidulans*: specific activities of ornithine δ-transaminase *pro⁺ SU⁺* (—○—) *pro⁺ SU-1* (—◐—) and *pro-6 SU-1* (—●—) strains.

of *su-2* locus slight repression was observed. The *pro-6* mutation itself caused an increase in OTA activity, as best indicated by comparison of the figures obtained for *pro-6 su-6* and *pro⁺ su-6* strains. The separate effect of the *pro-6* mutation was also seen by comparing the OTA activity in young cultures of strains of the *pro su* and *pro⁺ su* types, where *su* represents any mutation of the *su-2* locus (Fig. 1). No qualitative differences between the strains tested were observed in the response of OTA to the presence of ornithine. In all cases a marked decrease of activity was observed.

Table 3. *Aspergillus nidulans: specific activities of arginase in pro⁺ su⁺, pro su⁺ and pro⁺ su strains grown on minimal medium (MM) and media supplemented as indicated*

		Media			
		MM	+proline	+ornithine	+arginine
Suppressor locus	Genotype of strain	Arginase activity (mμ moles ornithine/mg. protein/min.)			
·	*pro⁺ su⁺*	11	6	51	560
	pro-6 su⁺	·	240	54	530
su-6	*pro⁺ su-6*	6	6	7	490
su-19	*pro-6 su-19*	51	332	246	595
su-2	*pro-6 su-2*	40	98	70	532
	pro-6 SU-1	64	150	52	520
	pro-6 SU-8	54	120	74	540
	pro⁺ su-2	42	48	125	505
	pro⁺ SU-1	72	52	101	489

Effects of suppressor mutations on arginase activity

Arginase was assayed in the wild strain and various mutant strains grown on minimal medium and on media supplemented with proline, ornithine or arginine. The results (Table 3) show that, in comparison to the wild type, arginase had lower activity in the *su-6* strain and about 4–6 times higher in the *su-2* and *su-19* strains. In the case of the *su-2* locus the increase of the enzyme activity was due to the presence of the suppressor mutations, as indicated by comparison of the figures for the strains of the *pro su* and *pro⁺ su* types. In the case of the *su-19* locus no strain of the *pro⁺ su* type was available (*pro-6* and *su-19* loci are closely linked which makes the selection of the *pro⁺ su-19* strain difficult) and therefore it is not clear whether the high activity of arginase in the *pro-6 su-19* strain should be ascribed to the *su-19* or to the *pro-6* mutation. The comparison of the figures obtained for the wild and *pro-6 su* strains grown in the presence of ornithine makes it obvious that the *pro-6* mutation must be taken into consideration when arginase activity is concerned. The exogenously supplied proline had a very strong effect on arginase activity, causing repression of the enzyme synthesis in the wild strain and a significant induction in all strains carrying the *pro-6* mutation.

Arginase activity was increased when the enzyme extracts were incubated before assay at 37° or 50°. When pre-incubation at 37° was used the activity of the enzyme reached a maximum after 15–20 min., and further pre-incubation caused no change in activity. At 50° the activity maximum was reached after 2–4 min. When manganese was omitted from the buffer used for extraction, activation did not occur. The factor by which pre-incubation increased the activity was very similar in all strains tested; one

set of results is given in Table 4. This being so, and since the pre-incubation results were not very reproducible, most of the work, including that reported in Table 3, was done without pre-incubation.

Table 4. *Aspergillus nidulans: specific activities of arginase in wild-type and suppressor strains after 30 min. pre-incubation at 37° and after 3 min. pre-incubation at 50°*

Genotype of strain	Pre-incubation: None	37°, 30 min.	50°, 3 min.
	Arginase activity (mμ moles ornithine/mg. protein/min.)		
pro⁺ su⁺	11	40	120
pro⁺ su-2	40	170	370
pro⁺ SU-1	72	290	560

Effects of suppressor mutations on arginine content in the mycelium

Free arginine content in the mycelium of the wild strain of *Aspergillus nidulans* and of suppressor strains was compared after separation of the amino acids of boiled mycelium extracts by high voltage electrophoresis and treating the resulting electrophoretogram with the arginine-specific stain according to the method of Irreverre (1965). The arginine spots seemed to be identical in intensity for all strains, with the exception of strains carrying *su-6* mutations where the colour obtained appeared to be markedly weaker (comparison by eye).

A second approach to this problem was to investigate canavanine resistance in the wild strain and in two suppressor strains, one carrying a dominant and the other one a recessive mutation at the *su-2* locus. It was assumed that strains producing more arginine should be more resistant to canavine, an arginine analogue, as the inhibition of growth caused by canavine is of a competitive type (Schwartz & Maas, 1960). The results are shown in Table 5. Expressing the inhibition as the factor by which canavanine decreased the mycelial weight in 24 hr cultures the figures are 4·0 for the wild strain, and 10·0 and 22·8 for recessive and dominant suppressor strains, respectively.

Table 5. *Aspergillus nidulans: the dry weight of mycelium of wild-type and suppressor strains obtained from cultures without and with canavanine*

Genotype of strain	Presence of canavanine (100 μg/ml.)	Time of incubation (hr): 24	34	46
		Mycelium (mg. dry wt.)		
pro⁺ su⁺	+	12·0	62·0	89·4
	−	47·6	225·0	390·0
pro⁺ su-2	+	2·4	42·0	99·6
	−	24·0	186·2	324·2
pro⁺ SU-1	+	2·1	30·1	120·0
	−	46·8	182·9	363·6

DISCUSSION

The modes of action of all suppressors studied in this work have one common feature, i.e. the suppressors all affect the pathway of arginine synthesis or arginine break-down in a way that permits the synthesis or proline by the alternative route (Fig. 2). In the case of the *su-6* mutations, which affect the ornithine transcarbamylase activity, this is achieved by decreasing the rate of arginine synthesis from ornithine and thus enhancing the accumulation of ornithine which in these circumstances

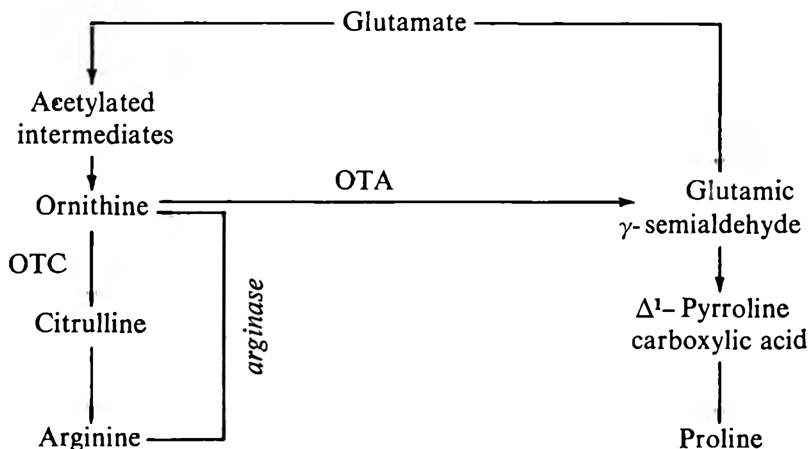

Fig. 2. *Aspergillus nidulans*: the pathway of proline and arginine synthesis. OTC, Ornithine transcarbamylase; OTA, ornithine δ-transaminase.

becomes available for transmutation to glutamic δ-semialdehyde in the proline pathway. The same mechanism of suppression was described by Davis (1962) in *Neurospora crassa*, where the suppressor *s*, suppressing some of the pyrimidine mutants and the proline mutants of the same type as those studied in this work, was the mutant affecting the level of ornithine transcarbamylase.

The *su-19* suppressor can be considered as the ornithine δ-transaminase regulator gene. Mutations at this locus cause the synthesis of the enzyme to be constitutive (in one case) or semiconstitutive (in two other cases studied). One can assume that there is a competition between ornithine transcarbamylase and ornithine δ-transaminase for ornithine which is entirely or almost entirely converted to citrulline by ornithine transcarbamylase in the wild strain, but partly converted to glutamic δ-semialdehyde by the highly active ornithine δ-transaminase in the *su-19* mutants.

The function of the *su-2* locus, mutation at which causes an increase of both arginase and ornithine δ-transaminase activities, may be explained in two ways. One possible explanation is that the *su-2* mutations lead to an increase in arginine production (caused, for example, by a failure in feedback inhibition of the arginine pathway by arginine) and that the higher activities of the arginine breakdown enzymes are due to induction by the endogenous arginine. The second possibility is that the primary effect of the mutation in the *su-2* locus is on the regulation of arginine break-down. The greater sensitivity of suppressor strains to canavanine and the failure to demonstrate

accumulation of arginine by suppressor strains seem to favour the second possibility for the function of the *su-2* locus. Whatever the primary effect of mutation in the *su-2* locus, the increase in arginine break-down to ornithine and then to glutamic δ-semialdehyde can explain the suppression of proline mutants in this case. The difference between dominant and recessive mutants of this locus lies most probably in the greater effectiveness of arginine break-down indicated by the higher arginase activity in strains carrying the dominant mutation.

The system of regulation of arginine break-down seems to be rather a complex one. The data obtained indicate the importance of proline or glutamic δ-semialdehyde in controlling the formation of arginase and ornithine δ-transminase. The effect of exogenous proline on that pathway, which was also observed in *Neurospora crassa* (Andersson-Kotto & Ehrensvard, 1963) and especially the different effect of proline on the arginase and ornithine δ-transaminase activities in wild strain and in proline-requiring strains, as well as the effects of ornithine on ornithine δ-transaminase activity cannot be satisfactorily explained at the moment.

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Biochemical and Genetic Studies with Regulator Mutants of the *Pseudomonas aeruginosa* 8602 Amidase System

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SUMMARY

Mutants of *Pseudomonas aeruginosa* strain 8602 were isolated which were unable to produce an aliphatic amidase (acylamide amidohydrolase, EC 3.5.1.4) and could not grow on acetamide as a carbon or nitrogen source. Amidase-constitutive mutants, producing amidase in the absence of inducing amides, were isolated by selection on succinate+formamide agar. Sixteen mutants were magno-constitutive non-inducible mutants producing amidase at about the same rate or greater than the fully induced wild-type strain. Amidase synthesis in one magno-constitutive mutant was repressed by the non-substrate inducer *N*-acetylacetamide, but the others were not affected in any way. Six mutants were semi-constitutive, producing amidase at 10–50 % of the rate of the magno-constitutive mutants and were induced by *N*-acetylacetamide. Most of the constitutive mutants were very sensitive to catabolite repression by succinate in pyruvate medium, but succinate produced only partial repression of one magno-constitutive mutant and three semi-constitutive mutants; one semi-constitutive mutant was not repressed except in the presence of inducer.

Six mutants isolated from succinate+formamide agar had altered inducer specificity and were induced to form amidase by formamide, which is a very poor inducer for the wild-type strain. The formamide-inducible mutants were also sensitive to catabolite repression by succinate although one mutant was only partially repressed.

Phage F 116 was used to transduce the amidase structural and regulator genes. In crosses between constitutive mutants of *Pseudomonas aeruginosa* as donors and amidase-negative mutants as recipients, the two characters were co-transduced with frequencies of 80–100 %. Similarly, in crosses between formamide-inducible and amidase-negative mutants these two characters were co-transduced with frequencies of 89–96 %. The amidase structural and regulator genes are considered to be closely linked.

INTRODUCTION

The aliphatic amidase (acylamide amidohydrolase, EC 3.5.1.4) produced by *Pseudomonas aeruginosa* 8602 is induced by growth in the presence of several substrate and non-substrate amides (Kelly & Clarke, 1962; Brammar & Clarke, 1964). Exponentially growing cultures synthesize the enzyme at a constant differential rate following a lag of about one generation after the addition of either acetamide (substrate inducer) or *N*-acetylacetamide (non-substrate inducer). Enzyme synthesis is repressed by certain acetamide analogues, e.g. cyanoacetamide and thioacetamide. Amidase synthesis is also subject to catabolite repression by propionate, acetate,

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succinate and some other metabolites (Brammar & Clarke, 1964). Amidase-negative mutants, which are unable to grow with acetamide as a carbon + nitrogen source, have been isolated after treating the wild-type strain with mutagenic agents (Skinner & Clarke, 1965). The genetic character for amidase production can be transferred by phage-mediated transduction using phage F 116 isolated by Holloway, Egan & Monk (1960). Constitutive mutants which produce amidase in the absence of inducer have been isolated (Clarke & Brammar, 1965), and the genetic character for constitutivity can also be transferred by phage F 116. We have examined the kinetics of enzyme synthesis for a number of the constitutive mutants and other regulator mutants with altered inducibility, and used phage transduction to investigate the linkage of the amidase structural and regulator genes. A brief report of part of this work has appeared previously (Clarke & Brammar, 1965).

Stanier, Palleroni & Doudoroff (1966) reported that 90 % of *Pseudomonas aeruginosa* strains can grow on acetamide and it seems probable that they all produce similar amidases.

METHODS

Organism. The parent strain was originally obtained from the National Collection of Type Cultures as *Pseudomonas aeruginosa* NCTC 8602. It was subcultured repeatedly in an acetamide-containing medium to enhance the acetamidase activity and the culture obtained was referred to as strain 8602/A (Kelly & Clarke, 1962). The National Collection have now discarded NCTC 8602 so that it can no longer be confused with our strain which we now refer to as *P. aeruginosa* strain 8602. Constitutive mutants are referred to as mutants C 1 etc.; amidase-negative mutants as mutants Am 1 etc.; formamide-inducible mutants as mutants F 1 etc. The pseudomonad phage F 116 isolated by Holloway *et al.* (1960) was kindly provided by Dr B. Holloway.

Maintenance of cultures. Stock cultures were maintained on slopes of nutrient agar prepared from Oxoid Nutrient Broth no. 2 powder and containing Oxoid no. 3 agar (1·2 %, w/v). The stock cultures were stored at 4° and mutant strains were lyophilized as soon as possible after isolation and identification.

Media for enzyme synthesis experiments. The media were based on the minimal salt medium described by Brammar & Clarke (1964). The differential rates of amidase synthesis and the inducibility ratios were measured in minimal salt medium containing 1 % (w/v) sodium succinate. Catabolite repression by succinate was measured in minimal salt medium containing 1 % (w/v) sodium pyruvate. Acetamide medium was prepared by adding a solution of acetamide sterilized by membrane filtration to the minimal salt medium to give a final concentration of 0·5 % (w/v).

Medium for plating out cultures. The minimal salt medium was used with 1·2 % (w/v) Oxoid no. 3 or Difco Noble agar. Carbon and nitrogen sources were added as follows for succinate agar (S plates) 1 % (w/v) sodium succinate; 0·1 % (w/v) ammonium sulphate; for acetamide agar (AM plates) 0·5 % (w/v) acetamide; for succinate + formamide agar (S/F plates) 1 % (w/v) sodium succinate + 0·1 % (v/v) formamide; for succinate + formamide + cyanoacetamide agar (S/F/CN plates) 1 % (w/v) sodium succinate + 0·05 % (v/v) formamide + 1 % (w/v) cyanoacetamide; for succinate + lactamide agar (S/L plates) 1 % (w/v) sodium succinate + 0·02 % (w/v) lactamide.

Dilution buffer. Before plating, the bacteria were resuspended and diluted in buffer pH 7·2 containing (%, w/v): 0·3 KH_2PO_4, 0·7 Na_2HPO_4, 0·4 NaCl and 0·02 $MgSO_4$.

Growth conditions. Cultures were grown for enzyme experiments in conical flasks of capacity 10 times that of the medium volume and shaken at 37° on a mechanical shaker. The culture medium was inoculated with 1/20 of its volume of an overnight culture in the same medium. Experiments on the rates of enzyme synthesis were started when the culture had become established in the exponential growth phase.

Growth measurements. The optical extinctions of the cultures were measured at 670 mμ with a Unicam SP 600 spectrophotometer. A standard curve was used to convert the readings to dry wt. bacteria.

Enzyme assays. Amidase activity was measured by the hydroxamate method for transferase activity described by Brammar & Clarke (1964). The substrate mixture contained 100 mM-acetamide, 500 mM-hydroxylamine hydrochloride, freshly neutralized to pH 7·2, and 50 mM-tris buffer (pH 7·2). The reaction was carried out in a total volume of 1 ml. and enzyme samples for assay were 0·5–0·25 ml. The reaction mixture was incubated in a water bath at 37°; the time of incubation was varied between 5 and 20 min. according to the activity of the sample. The specific activity was defined as μmoles acethydroxamate produced/mg. dry wt. bacteria/min.

Amides. Acetamide was obtained from Hopkin and Williams Ltd. and recrystallized twice from ethanol. *N*-acetylacetamide was prepared by refluxing acetamide with excess acetic anhydride, removing the residual acetic anhydride by distillation and twice recrystallizing the product from ethylmethyl ketone. Cyanoacetamide was obtained from Hopkin and Williams Ltd. and purified by recrystallizing twice from methanol. Lactamide, prepared by ammoniolysis of lactide, was a gift from Dr P. Draper. Formamide was stored in the dark.

Reagents. Hydroxylamine hydrochloride was recrystallized 3 times from distilled water. Tris (2-amino-2-hydroxymethylpropane-1,3-diol) was recrystallized twice from methanol + water (20 + 1 by vol). Ethylmethane sulphonate (EMS) was obtained from Kodak Ltd., London. *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NMG) was obtained from the Aldrich Chemical Co.

Mutagenic treatment: ethylmethane sulphonate (EMS) as mutagen. EMS was added to a 5 hr culture of wild-type strain 8602 growing either in nutrient broth or succinate medium to give a final concentration of 160 mM. The culture was incubated at 37° for 15 min. or at room temperature for 1 hr, centrifuged, the deposit washed twice and resuspended in dilution buffer.

N-methyl-N'-nitro-N-nitrosoguanidine (NMG) as mutagen. NMG was added at a final concentration of 200 μg./ml. to a 5 hr nutrient broth culture of the wild-type strain resuspended in 100 mM citrate buffer (pH 6·0). The cultures were incubated for 75 min. at room temperature (0·1 % survival), centrifuged, the deposit washed twice and resuspended in dilution buffer.

Ultraviolet irradiation. Bacteria from a 5 hr broth culture were resuspended in dilution buffer and irradiated at 2537Å (95 %) for 2 min. at a distance of 12 cm. (I = 854 ergs/cm.^{2}/sec.). These conditions gave about 0·1 % survival.

Selection of mutants. To obtain amidase-negative (Am$^-$) mutants, 0·1 ml. samples of treated bacteria were inoculated into nutrient broth, grown overnight, diluted to give 10^3 bacteria/ml. and 0·1 ml. spread on each of about 60 plates. Amidase-negative mutants were selected as small colonies on acetamide plates, or acetamide plates supplemented with 0·001 % (w/v) sodium succinate.

Regulator mutants were obtained by plating about 10^8 bacteria on S/F plates either

immediately after mutagenic treatment, or after overnight growth in nutrient broth. The mutants grew as large colonies from a faint background growth of wild-type bacteria. These colonies were picked off, grown overnight in succinate medium and tested for amidase activity. To eliminate wild-type bacteria, the mutant cultures were replated on S/F plates to give about 20 colonies/plate, and single mutant colonies isolated.

Preparation of phage lysates. The preparation of high-titre lysates of phage F 116 followed the method of Adams (1959). A broth culture of bacteria was mixed with phage, and 0·2 ml. samples containing 3×10^8 bacteria and 10^6–10^7 plaque forming units (p.f.u.) were spread on each nutrient agar plate. Incubation for 18 hr at 37° resulted in confluent lysis. The phage was harvested in TNM buffer (pH 7·1), consisting of 10 mM-tris, 10 mM-magnesium sulphate and 150 mM-sodium chloride. Phage stocks were sterilized by adding chloroform, after low-speed centrifugation to remove bacterial debris, and stored at 4°. They were assayed by the agar layer technique. The transducing phage was propagated twice in the donor strain to eliminate the wild-type phage particles. This method consistently produced 10^{10}–10^{11} p.f.u./ml.

Transduction. The procedure for transduction followed that of Holloway, Monk, Hodgins & Fargie (1962). Recipient bacteria were grown overnight in nutrient broth, centrifuged down and resuspended in dilution buffer to give 2–4×10^9 bacterial/ml. Samples (1 ml.) were mixed with 1 ml. phage suspension at a multiplicity of 5 to 10. After 60 min. at 37° for phage adsorption, the bacteria were centrifuged down and resuspended in 2 ml. dilution buffer. Samples (0·2 ml.), diluted when necessary, were spread over the surface of agar plates to give 100 to 150 transductants/plate after 48–72 hr incubation at 37°. Controls were included to detect reversion of recipient bacteria, by plating 0·1 ml. samples of resuspended bacteria on appropriate media. In addition, 0·1 ml. samples of phage were plated on nutrient agar to detect viable bacteria in the phage stock.

Replica plating. Transductants were replicated after 72 hr incubation according to Lederberg & Lederberg (1952).

RESULTS

Isolation of regulator mutants

A method was devised which depended on using a non-inducing amide as a growth substrate so that any constitutive mutants would have a selective advantage over the wild-type strain. The most suitable amide for this purpose was formamide, but as this strain of *Pseudomonas aeruginosa* is unable to grow with one-carbon compounds as sole carbon source, the medium contained succinate as carbon source and formamide as nitrogen source (S/F plates). The wild-type strain produced only faint shadowy growth on this medium so that the mutants appeared as large colonies growing out of a background haze.

The specific activities of the mutant cultures after overnight growth in succinate medium varied over a wide range. Some mutants behaved like the wild-type parent and produced little or no amidase activity while others produced as much enzyme as a fully induced wild-type culture. This procedure therefore appeared to select not only constitutive mutants but also a class of inducible mutants which, unlike the parent

wild-type, were able to grow on S/F plates. Spontaneous mutation to allow growth on S/F plates occurred in $1/10^7$ to $1/10^6$ bacteria and the rate was increased by mutagenic treatment.

Properties of constitutive mutant C 1

The first constitutive mutant identified was compared with the wild-type strain with respect to growth rate and factors affecting the rate of amidase synthesis. There was no significant difference in the growth rates of the two cultures in nutrient broth or minimal salt media. In succinate medium the differential rate of amidase synthesis was equal to or slightly greater than that of the fully induced wild type.

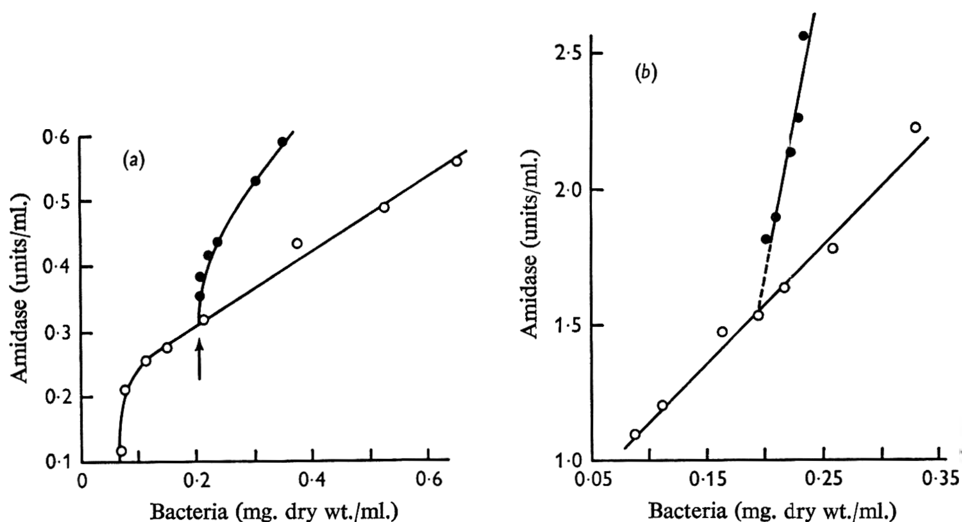

Fig. 1*a*. The effect of azide on amidase synthesis by the wild-type strain of *Pseudomonas aeruginosa* 8602. ●—●, 2 mM-sodium azide added at the point indicated by arrow; ○—○, control culture; acetate medium used for experiment.

Fig. 1*b*. The effect of azide on amidase synthesis by the constitutive mutant of *Pseudomonas aeruginosa* 8602 C 1. ●—●, 5 mM-sodium azide added at point indicated by arrow; ○—○, control culture; succinate medium used for experiment.

One of the characteristic features of amidase synthesis by the wild type in acetamide medium was the second phase of enzyme synthesis as the culture reached the end of exponential growth (Kelly & Kornberg, 1962). This late-phase synthesis was also found with acetate as the growth substrate but with no other carbon source tested. Amidase synthesis in acetate medium occurred earlier when the pH value of the medium was allowed to increase. This effect appeared to be related to a decrease in the growth rate and was also produced by the addition of sodium azide to an exponnentially growing culture. In the experiment shown in Fig. 1*a* the growth rate was decreased by about 40 % and there was marked stimulation of the differential rate of amidase synthesis. The constitutive mutant C 1 synthesized amidase at a constant differential rate during exponential growth in succinate medium but there was a marked increase in the rate of enzyme synthesis towards the end of the growth period. Figure 1*b* shows the effect of azide on amidase synthesis during exponential growth. In this experiment it produced a 4-fold decrease in the growth rate and a 4-fold

increase in the differential rate of amidase synthesis, so that the absolute rate was unaffected.

The non-substrate inducer *N*-acetylacetamide was used to test mutant C 1 for inducibility. At a concentration of 10 mM, which is saturating for induction of the wild-type strain, it had no effect on amidase synthesis by mutant C 1 at any stage of growth. Mutant C 1 therefore behaved as a fully constitutive or non-inducible strain. According to the classification proposed by Collins *et al.* (1965) it could be described as a strictly constitutive magno-constitutive strain, since it produced amidase at about the same rate as the fully induced wild-type strain in the presence or absence of inducer.

Many normal cell metabolites can cause catabolite repression of amidase synthesis by the wild-type strain. Acetate and propionate repress amidase induction by acetamide or *N*-acetylacetamide in succinate medium (Brammar & Clarke, 1964). Succinate

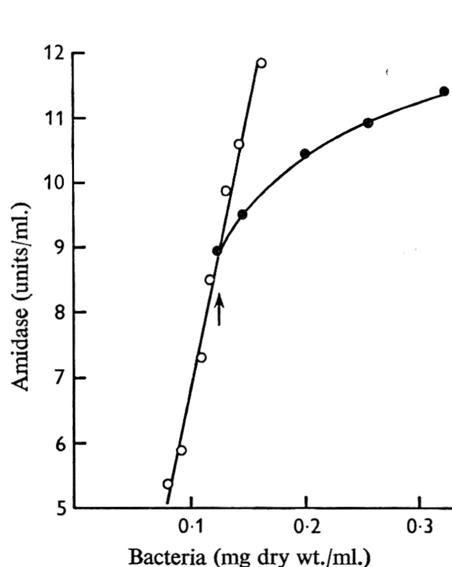

Fig. 2

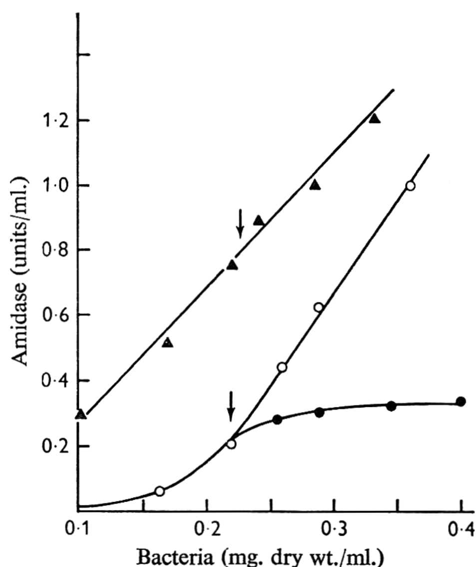

Fig. 3

Fig. 2. Catabolite repression by succinate of amidase synthesis by the constitutive mutant of *Pseudomonas aeruginosa* 8602 C 1. ●—●. 10 mM-sodium succinate added at point indicated by arrow; ○—○ control culture; pyruvate medium used for experiment.

Fig. 3. Effect of cyanoacetamide on amidase synthesis by constitutive and wild-type strains of *Pseudomonas aeruginosa* 8602. Wild-type strain induced with 10 mM-*N*-acetylacetamide; ●—●, 10 mM-cyanoacetamide added at point indicated by arrow; ○—○, control culture; ▲—▲, mutant C 1 10 mM-cyanoacetamide added at point indicated by arrow.

also exerts catabolite repression, e.g. amidase induction by 10 mM-*N*-acetylacetamide in pyruvate medium is 98–100 % repressed by the addition of 10 mM succinate to growing cultures. Figure 2 shows that mutant C 1 growing in pyruvate medium was like the wild-type strain, very sensitive to repression by succinate.

We had concluded previously from experiments with growing cultures (Brammar & Clarke, 1964) and carbon-starved bacteria (Clarke & Brammar, 1964) that the amide analogue cyanoacetamide repressed amidase synthesis by competing with the inducer for an amide-specific binding site. It has now been shown (Brammar, McFarlane & Clarke, 1966) that competition for a permease is not involved since cyanoacetamide

had no effect on the uptake of ^{14}C-labelled *N*-acetylacetamide at a concentration which prevented amidase induction. If the site of competition of the amides is the inducer-binding site of a cytoplasmic repressor molecule it could be predicted that at least some of the constitutive mutants would be unaffected by the amide analogue. Figure 3 shows that 10 mM-cyanoacetamide almost completely repressed amidase synthesis by an induced wild-type culture and had no effect on the synthesis of amidase by mutant C 1.

Survey of constitutive mutants

The constitutive mutant C 1 had been tested in considerable detail to compare it with the wild-type strain. Similar but slightly simplified methods were used to examine the other regulator mutants. The enzyme studies were carried out with exponentially growing cultures and about 5 samples were taken over a period of 1·5–2·5 hr, depending on the growth conditions.

Differential rates of amidase synthesis. The measurements were made in succinate medium and were calculated as a percentage of the rate for mutant C 1. There was some variation in the rate of amidase synthesis by mutant C 1 in different experiments which was thought to be due to slight variations in the growth conditions. Each mutant culture was therefore tested in parallel with mutant C 1. Several mutants were tested on two or three occasions and gave results agreeing within 5 %. Figure 4 shows a typical experiment and compares the differential rates of amidase synthesis by mutants C 8, C 11, C 20 with mutants C 1. The results for 22 constitutive mutants tested are given in Table 1. Most of these mutants had a differential rate of amidase synthesis in succinate medium within 80–120 % of that of C 1. A few had higher rates; C 11 and C 24 were the highest with a rate of 165, 6 mutants had significantly lower rates ranging from 10 % to 50 % of that of C 1.

Inducibility. Preliminary experiments compared the specific activities of organisms grown overnight in succinate medium with those of organisms grown in succinate medium containing 10 mM-*N*-acetylacetamide as non-substrate inducer. The results suggested that the mutants with high differential rates of amidase synthesis like C 1 were not induced to form more amidase in the presence of *N*-acetylacetamide, but that some of the mutants with low differential rates were partially inducible. This was investigated further by using exponentially growing cultures. Soon after the cultures had reached the exponential growth phase they were divided into two parts to one of which *N*-acetylacetamide was added to a concentration of 10 mM. The results are summarized in Table 1. The constitutive mutants with differential rates of amidase synthesis which were 80 % or more of that of C 1 were not induced to synthesize amidase at an increased rate by *N*-acetylacetamide. They therefore behaved as fully constitutive or non-inducible strains. Mutant C 24 was unusual in that amidase synthesis was repressed 68 % by 10 mM-*N*-acetylacetamide.

The six constitutive mutants with lower differential rates of amidase synthesis were all inducible, i.e. semi-constitutive. The inducibility ratio was calculated as

$$\frac{\text{rate of amidase synthesis with 10 mM-}N\text{-acetylacetamide}}{\text{rate of amidase synthesis without inducer}}.$$

The values obtained were mostly 2 to 3; mutant C 9 with a differential rate of 10 to 14 had an inducibility ratio of 9, but this culture could be clearly distinguished from the wild-type strain which had an inducibility ratio of > 100 under these conditions.

Catabolite repression. Mutant C 1 had been found to be almost as sensitive as the wild-type strain to catabolite repression by succinate in pyruvate medium. Similar experiments were done with the other constitutive mutants. All but one of the magno-constitutive mutants were repressed 80–90 % by 10 mM-succinate. C 24 was again the exception among the magno-constitutive mutants and was only repressed by 63 %.

Table 1. *Properties of amidase constitutive mutants of Pseudomonas aeruginosa 8602*

Mutant	Mutagen*	Differential rate†	Induction ratio‡	Catabolite repressibility§	Phenotype
C 1	EMS	100	1	96	Magno-constitutive
C 4	NMG	130	1	80	
C 6	NMG	100	1	85	
C 7	NMG	96	1	80	
C 8	NMG	83	1	80	
C 10	NMG	96	1	85	
C 11	Spontaneous	165	1	85	
C 12	UV	114	1	87	
C 13	UV	113	n.t.	83	
C 14	UV	107	n.t.	88	
C 16	UV	90	n.t.	80	
C 18	Spontaneous	100	1	n.t.	
C 19	Spontaneous	100	1	n.t.	
C 21	EMS	137	1	84	
C 22	EMS	129	1	84	
C 24	EMS	165	0·3	63	
C 2	NMG	19	2	90	Semi-constitutive
C 5	NMG	23	3	0	
C 9	NMG	10–14	9	55	
C 15	UV	39	3	55	
C 17	UV	47	n.t.	80	
C 20	EMS	32	2	93	

n.t. = not tested.

* Mutagens. EMS = ethylmethane sulphonate; NMG = *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine, UV = ultraviolet irradiation.

† Differential rate: the rate of amidase synthesis/mg. bacterial growth for mutant C 1 in succinate medium was assigned a value of 100. The results for all other mutants are expressed as a percentage of that for C 1.

‡ Induction ratio: the differential rate of amidase synthesis in succinate medium containing 10 mM-*N*-acetylacetamide/rate of synthesis in the absence of inducer.

§ Catabolite repressibility: the percentage repression of the rate of amidase synthesis in pyruvate medium in the presence of 10 mM-sodium succinate.

The semi-constitutive mutants were more variable in their sensitivity to catabolite repression. C 2, C 17 and C 20 were as sensitive to catabolite repression as the magno-constitutive mutants, but mutants C 9 and C 15 were repressed only 55 % and C 5 was not repressed at all. The semi-constitutive mutants repressible by succinate synthesized amidase constitutively in pyruvate medium at least three times faster than in succinate medium, but the rate of amidase synthesis by C 5 was the same in both media. With 10 mM-*N*-acetylacetamide added as inducer in pyruvate medium, the total rate of amidase synthesis by mutant C 5 (constitutive + induced) was repressed by 56 %.

Formamide-inducible mutants

The mutants isolated from S/F plates which did not produce amidase constitutively nevertheless required amidase for growth, since formamide was the only nitrogen source provided in the medium. It seemed likely that these mutants differed from the wild type in being induced by formamide. They were grown overnight in liquid succinate medium containing 0·1 % (w/v) formamide and were found to produce low but detectable amounts of amidase. When the induction rates were measured with growing cultures and formamide, acetamide or propionamide as inducers, the mutants differed from the wild type in their inducibility patterns for the three amides. There

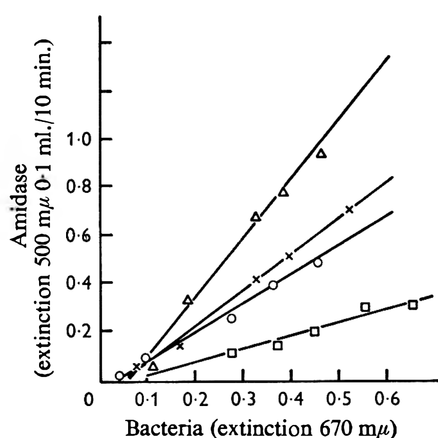

Fig. 4

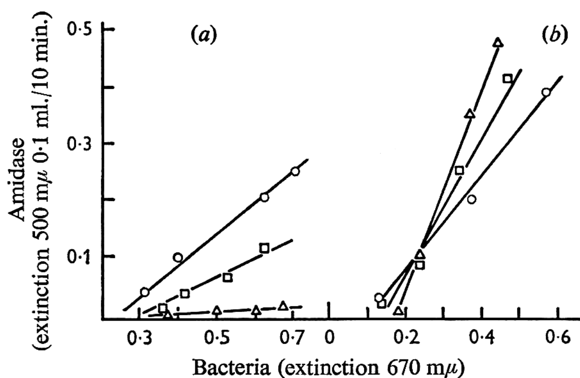

Fig. 5

Fig. 4. Differential rates of amidase synthesis by constitutive strains of *Pseudomonas aeruginosa* 8602. ×—×, Strain C 1; ○—○, strain C 8; □—□, strain C 20; △—△, strain C 11.

Fig. 5. Induction of amidase synthesis by formamide (△—△), acetamide (○—○) and propionamide (□—□), with (*a*) the wild-type strain and (*b*) mutant F_6. Amides added at 10 mM in succinate medium.

was no significant difference in the basal rate of amidase synthesis in the absence of inducer between the wild type and any of the F mutants. Although it had previously been thought from experiments with cultures grown overnight that formamide was not an inducer (Kelly & Clarke, 1962), these experiments showed that it had weak inducing activity for the wild-type strain and significantly higher inducing activity for all these mutant strains. Figure 5 compares the differential rates of amidase induction by the three amides for the wild-type and mutant F 6 which had the most marked alteration in amide inducibility pattern. It can be seen that for this mutant formamide was a more effective inducer than acetamide under these conditions. The results for all the F mutants tested are given in Table 2.

Since formamide is a weak inducer for the wild-type strain it would be more effective in succinate medium if a mutation occurred which resulted in a decreased sensitivity to catabolite repression by succinate. Although the previous experiments suggested that the F mutants had mutations which affected the inducer specificity, it was thought that catabolite repressibility could have been altered at the same time. The cultures were induced with *N*-acetylacetamide in pyruvate medium and tested for

catabolite repression by 10 mM-succinate. The results (Table 2) showed that although the sensitivity to catabolite repression of these strains was less than that of the wild type the differences were not very great, except for mutant F 6 which was only 60 % repressed.

Table 2. *Properties of formamide-inducible mutants of Pseudomonas aeruginosa 8602*

Mutant strain	Inducer: Formamide	Inducer: Acetamide	Inducer: Propionamide	Catabolite repressibility†
	Rate of amidase induction*			
Wild type	0·3	4·2	2·2	98–100
F 1	3·8	4·9	7·5	60
F 2	3·1	6·4	4·8	84
F 5	1·2	3·2	2·2	86
F 6	10	4·7	7·3	88

* Rate of amidase induction. Amidase units synthesized/mg. bacterial growth.

† Catabolite repressibility. The percentage repression of the rate of amidase synthesis induced by 10 mM-*N*-acetylacetamide in pyruvate medium resulting from the addition of 10 mM-succinate.

Table 3. *Growth of Pseudomonas aeruginosa 8602 wild type and mutant strains on selective media*

Growth after incubation for 48 hr at 37° is scored as follows: + + + +, colonies raised, usually with yellow fluorescent pigment; +, colonies very small or faint, no pigmentation; trace, very faint shadowy colonial growth. Details of media in methods section.

Mutant strain	Media containing: Acetamide	Succinate + formamide	Succinate + formamide + cyanoacetamide	Succinate + lactamide
Wild type	+ + + +	Trace	Trace	+
C 1	+ + + +	+ + + +	+ + + +	+
C 2	+ + + +	+ + + +	+	+
C 5	+ + + +	+ + + +	+	+ + + +
C 24	+ + + +	+ + + +	+	+
F 1	+ + + +	+ + + +	Trace	+
Am 1	–	–	–	–

Growth of mutants on selective media

The constitutive and formamide-inducible mutants were distinguished from the wild type by their ability to grow on S/F plates. Other selective media were devised which can be used to distinguish other mutant classes.

Cyanoacetamide completely represses amidase induction and growth in liquid acetamide medium (Brammar & Clarke, 1964) but it was not possible to reach a sufficiently high ratio of cyanoacetamide to acetamide in solid medium to repress growth of the wild-type strain on plates. It was however possible to repress growth and amidase induction when the inducing amide was formamide. All the magno-constitutive mutants with the exception of C 24 grew as well on succinate + formamide + cyanoacetamide agar (S/F/CN plates) as on S/F plates. Growth of all the semi-constitutive mutants was delayed, and colonies took 4–5 days to develop whereas on S/F

plates they appeared in 24–48 hr. This suggests that growth of the semi-constitutive mutants on S/F medium normally involves amidase induction. Growth of all the formamide-inducible mutants was repressed on S/F/CN plates; this medium could therefore be used to select the magno-constitutive mutants.

Lactamide is an inducing amide with an activity similar to that of propionamide. It was previously reported (Kelly & Clarke, 1962) that it was not a substrate for the enzyme, but Kelly & Kornberg (1964) found that a preparation of their partially purified enzyme had an activity of 23·8 units/mg. protein for lactamide as compared with 260 units/mg. protein for acetamide. We have used a minimal agar medium containing 1 % (w/v) succinate and 0·02 % (w/v) lactamide in mutant selection experiments. On this medium the wild type and almost all the regulator mutants grew very slowly, producing colonies in 5–7 days. Strain C 5 grew very well in 24–48 hr and C 9 grew significantly faster than the other mutant strains. This phenotype appears to be correlated with decreased susceptibility to catabolite repression. Table 3 summarizes the growth characteristics of the mutants on these selective media.

Genetic analysis by transduction

Preliminary experiments with a lysate of bacteriophage F 116 propagated on the wild-type strain showed that the amidase gene could be transferred to seven amidase-negative (Am^-) mutants. The number of amidase-positive (Am^+) transductants obtained depended on the particular recipient used. The genetic character of constitutivity was also transferred by phage F 116. A lysate prepared with mutant C 1 and used to infect the inducible wild-type strain gave constitutive transductants on S/F plates after incubation for 48 hr at 37°. Transduction of the amidase and constitutivity markers occurred at similar frequencies (200 to 250 transductants/10^9 recipient bacteria).

When the phage lysate from mutant C 1 was used to infect the amidase-negative mutant AM 9, transductants were obtained on both acetamide and S/F plates. The occurrence of approximately equal numbers of colonies on both media suggested that the gene which conferred constitutivity was closely linked to the amidase structural gene and that these two markers had been co-transduced. Phage propagated on the inducible wild-type strain gave transductants only on acetamide (AM) plates, i.e. only inducible transductants. Infection of other Am^- mutants with the lysate prepared on mutant C 1, followed by selection of Am^+ transductants on AM and S/F plates, indicated 75–100 % linkage of the constitutivity marker to the amidase marker. This close linkage was determined more accurately by selecting Am^+ transductants on AM plates and replicating to S/F plates. When the transductants had also inherited constitutivity, they grew on S/F plates after 48-hr incubation at 37°. The results given in Table 4 show that 87 to 98 % of the Am^+ transductants carried the constitutivity marker associated with the C 1 donor. They also indicate that all 10 Am^- mutations were located in a small region of the bacterial chromosome. A lysate prepared on C4, another magno-constitutive mutant, gave identical linkage values (Table 4). Since all the Am^- mutants behaved similarly with respect to their linkage to the constitutivity marker carried by mutants C 1 and C 4, further transduction experiments with the remaining constitutive mutants were made with only two or three Am^- recipient mutants. Table 5 gives the results for a number of magno-constitutive and semi-constitutive mutants. They all resembled mutants C 1 and C 4 in that the constitutivity and amidase markers were co-transduced giving linkage values of 80–100 %.

After replicating Am^+ transductants to S/F plates, the time necessary for colonies to appear varied from 1 to 5 days of incubation at 37°, depending on the constitutive mutant used as donor. For example, Am^+ transductants obtained from mutant C 1 as donor grew on S/F plates after 24 hr while Am^+ transductants from a C 2 donor required 5 days of incubation after replicating. When Am^+ transductants were selected directly on S/F plates, a similar variation was observed in the time taken before transductant colonies appeared. This slow growth of transductants occurred when the donor was a semi-constitutive mutant. It was therefore necessary to confirm that such transductants were constitutive, rather than wild-type inducible growing out of the

Table 4. *Phage transduction from constitutive mutants to amidase negative mutants of Pseudomonas aeruginosa 8602*

Donor strain phage lysate	Recipient strain	No. of Am^+ transductant colonies examined	Constitutive transductants (%)
C 1 Magno-constitutive	Am 1	54	92
	Am 2	92	87
	Am 3	141	94
	Am 4	169	88
	Am 5	63	91
	Am 6	318	94
	Am 7	178	98
	Am 8	187	98
	Am 9	126	98
	Am 10	224	95
C 4 Magno-constitutive	Am 1	40	88
	Am 2	100	95
	Am 3	124	98
	Am 4	106	98
	Am 5	127	98
	Am 6	187	94
	Am 7	130	94
	Am 8	152	97
	Am 9	143	99
	Am 10	84	96

background growth of Am^- recipients. Single transductants colonies from either AM or S/F plates were isolated and tested. Cross-feeding of inducible bacteria by constitutive bacteria occurred readily so that it was not possible to identify them simply by streaking on S/F agar. The following procedure was adopted to detect the presence of the constitutivity marker in the Am^+ transductant. Single colonies were grown overnight in broth, the culture was then suitably diluted and a loopful added to a suspension of wild-type inducible bacteria (10^8/ml.) Samples (0·1 ml.) of the mixture were plated on S/F plates and incubated at 37°. If 200–250 colonies grew out of the background growth of wild-type bacteria after 1–4 days, it was concluded that the Am^+ transductant also carried the constitutivity marker of the donor. This test was applied to at least five transductants from each cross; it was also applied to all colonies which did not grow when replicated from AM to S/F plates. The results were always conclusive in distinguishing between constitutive and inducible Am^+ transductants. Transductants obtained by infecting Am^- mutants with phage propagated on the wild-type

inducible strain, produced only inducible Am^+ transductants which gave no detectable colonies in the wild-type background growth, even after 7 days of incubation.

Genetic analysis by transduction was also done with F mutants as donors. The method was the same as that used with the constitutive strains, but with the F mutants as donors growth on S/F plates indicated that formamide-inducible and not constitutive transductants had been selected. The results given in Table 5 show that the character for formamide inducibility was also co-transduced at high frequency with the character for amidase production.

Table 5. *Transduction of amidase structural and regulator genes in Pseudomonas aeruginosa 8602 mutants*

Donor strain phage lysate	Phenotype	Recipient strain	No. of Am^+ transductant colonies examined	Transductants growing on S/F* plates (%)
C 8	Magno-constitutive	Am 9	194	96
		Am 10	268	92
C 9		Am 7	105	92
		Am 10	100	93
C10		Am 9	183	99
		Am 10	257	99
C 2	Semi-constitutive	Am 7	68	97
		Am 10	109	97
C 5		Am 9	143	88
		Am 10	290	93
C 20		Am 7	246	97
		Am 10	244	95
F 1	Formamide-inducible	Am 7	242	94
		Am 10	161	93
F 2		Am 7	84	89
		Am 16	206	91
F 3		Am 7	237	96
		Am 10	266	96

* S/F plates = succinate+formamide agar plates.

DISCUSSION

The regulator mutants of *Pseudomonas aeruginosa* 8602 can be classified phenotypically by their response to the various substrate and inducer amides, in terms of enzyme production and enzyme activity. The amidase-negative mutants do not grow on acetamide or any other substrate amide and do not produce an active amidase under any conditions. We have assumed provisionally that they all have mutations in the structural gene which determine the amidase protein. We are investigating these further by more detailed genetic mapping and by testing for the production of mutant protein without enzymic activity.

Most of the constitutive mutants can be assigned to the group described by Collins *et al.* (1965) as 'strictly constitutive, induction ratio 1·0, subgroup (a) magno-constitutive'. This appears to be the most common class of constitutive mutant isolated in other systems, e.g. *Bacillus licheniformis* penicillinase (Dubnau & Pollock, 1965). One mutant, C 24, of *Pseudomonas aeruginosa* 8602 which was classed as magno-

constitutive from its rate of amidase synthesis in succinate medium in the absence of inducer, had an induction ratio of 0·3 when tested with *N*-acetylacetamide. Collins *et al.* (1965) discussed the possibility of the occurrence of this type of mutant, repressible by the normal inducer, and suggested that it should be considered with the class of repressible enzyme systems. We think that this would give a misleading impression of a mutant derived from a normally inducible enzyme system and prefer the description of magno-constitutive, inducer-repressible, for this strain. Jacob & Monod (1963) referred to a class of partially constitutive β-galactosidase mutants repressible by galactosides which were inducers for the parent strain. The other group of amidase-constitutive strains of *P. aeruginosa* 8602 are adequately described as semi-constitutive by using the nomenclature of Collins *et al.* (1965).

The mutants of *Pseudomonas aeruginosa* 8602 inducible by formamide are perhaps the most interesting and represent a mutant class which has not been previously described. They appear to have altered inducer specificity and a suitable general term to describe this phenotype would be 'neo-inducible' followed by an indication of the main change in inducer response, i.e. in our system 'neo-inducible, formamide-inducible'. There was no obvious difference in experiments with intact bacteria between the amidase produced by these strains and that produced by the wild-type strain and it was therefore assumed that the genetic change was in the regulator gene and not the structural gene. These mutants might be predicted from the Jacob & Monod (1961) theory of the regulation of enzyme synthesis. The mutations are probably in a regulator gene, resulting in the production of cytoplasmic repressor molecules with altered specificity towards the various amide inducers. By the same reasoning it is likely that the constitutive mutant C 24 of *P. aeruginosa* 8602 produces an altered cytoplasmic repressor which becomes effective in repressing amidase synthesis when combined with *N*-acetylacetamide, a non-substrate inducer for the wild-type strain. It is probable that the other magno-constitutive mutants are also regulator gene mutants in which the cytoplasmic repressor is altered or absent. The semi-constitutive mutants produce more enzyme in the presence of inducer than in its absence, and must therefore contain cytoplasmic repressor molecules which are capable of combining with the inducer. Our experiments cannot distinguish mutants of this phenotype which have mutations in a regulator gene (R mutants) from those which have mutations in an operator region (0 mutants). Linkage analysis from the results for co-transduction has shown that all the amidase regulator mutations are located in a small segment of the *P. aeruginosa* 8602 chromosome closely linked to the amidase structural gene. The amidase system therefore resembles the *lac* region of *Escherichia coli* where the R gene is closely linked to the structural genes (Jacob & Adelberg, 1959) rather than the tryptophan synthetase system where the R gene is located on a distant segment of the bacterial chromosome (Cohen & Jacob, 1959).

Regulator and operator-type mutations for β-galactosidase have been distinguished by examining the properties of diploids and by identifying operator mutations affecting the synthesis of the three separate proteins determined by the β-galactosidase operon, β-galactosidase, permease and transacetylase (Jacob & Monod, 1961). We have not so far been able to make diploids for our Pseudomonas system and we have no evidence that more than one structural gene is concerned. Recently it was found that this strain of *Pseudomonas aeruginosa* produced a permease for aliphatic amides (Brammar *et al.* 1966), but this was constitutive and it is not known whether there is

any linkage with the amidase genes. From transduction experiments with other mutants it was concluded that the amidase genes were not closely linked to genes which determined enzymes concerned with acetate metabolism, i.e. isocitrate lyase, acetyl-CoA synthetase and citrate synthetase.

Amidase synthesis by the wild-type strain *Pseudomonas aeruginosa* 8602 is very sensitive to catabolite repression, which could be reversed both in growing cultures and in carbon-starved bacteria, by increasing the inducer concentration (Brammar & Clarke, 1964; Clarke & Brammar, 1964). Most of the amidase constitutive mutants were also sensitive to catabolite repression and like the inducible strains had a maximum rate of amidase production in pyruvate medium at least three times higher than that in succinate medium. This suggests that succinate is a better source than pyruvate of the actual co-repressor molecule. It was shown in other systems that the addition of metabolic inhibitors such as 2,4-dinitrophenol (Mandelstam, 1961) or the introduction of anaerobic conditions (Cohn & Horibata, 1959) released the synthesis of several enzymes from repression; several degradative enzymes (e.g. amino-acid decarboxylases Gale, 1946) are produced only at the end of growth as the metabolic rate decreases. The addition of azide stimulated amidase synthesis both by the wild-type strain of *P. aeruginosa* 8602 and by the constitutive mutant C 1, probably by decreasing the metabolic rate and the availability of the usual catabolite co-repressor.

If catabolite repression is in any way related to the activity of the cytoplasmic repressor produced by the regulator gene it should be reversed by the inducer. This reversibility is now established for β-galactosidase in *Escherichia coli* (Clark & Marr, 1964; Moses & Prevost, 1966) and the enzymes of the mandelate pathway in *Pseudomonas fluorescens* (Mandelstam & Jacoby, 1965) as well as for *P. aeruginosa* amidase. It should also be possible to isolate regulator gene mutants with altered catabolite repressibility. McFall (1964) described several mutants for D-serine deaminase in *E. coli* which were partially constitutive and had altered sensitivity to catabolite repression. One magno-constitutive amidase mutant of *P. aeruginosa* 8602 had decreased sensitivity to catabolite repression and since it was also repressed by *N*-acetylacetamide it seems reasonable to suppose that it produced a cytoplasmic repressor which had altered response to the normal inducer. Several of the semi-constitutive mutants of *P. aeruginosa* 8602 had altered catabolite repressibility and mutant C 5 was repressed by succinate only in the presence of an inducer. Repression by succinate in the amidase system of *P. aeruginosa* 8602 has been shown to be quite different from the competitive repression by amide analogues such as cyanoacetamide (Clarke & Brammar, 1964) so that there is no possibility here of succinate competing with the inducer as Loomis & Magasanik (1966) suggested for galactose repression of β-galactosidase.

We suggested previously that the cytoplasmic repressor could be directly involved in catabolite repression by being able to combine with both inducer and catabolite co-repressor molecules with opposite effect (Clarke & Brammar, 1964). The results of Loomis & Magasanik (1964, 1965, 1966) and Moses & Prevost (1966) indicate that the mechanism may be more complex. A complete explanation of catabolite repression would depend on a fuller understanding of the molecular events which lead through transcription of genetic information of the DNA into RNA messengers to translation into specific proteins.

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The Growth of *Mycoplasma bovigenitalium* in Cell Cultures

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SUMMARY

A strain of *Mycoplasma bovigenitalium*, designated M 120, was grown, and produced a marked cytopathic effect (c.p.e.) in calf-, pig-, and monkey-kidney cell cultures. The c.p.e. was characterized by enlargement of the cells, the appearance of intracytoplasmic inclusions and partial destruction of the cell monolayers. A similar c.p.e. was produced in tissue-culture cells following the inoculation of mycoplasma 'toxins'.

Comparative growth studies of strain M 120 in calf-kidney cell cultures and in tissue-culture medium alone showed that the organism grew more readily in the presence of cells. The rate of virus production and appearance of c.p.e. of infectious bovine rhinotracheitis (I.B.R.) virus was delayed in cultures previously infected with the M 120 strain of mycoplasma as compared with normal cultures.

INTRODUCTION

Hayflick & Stinebring (1955) first reported the growth of human and avian strains of mycoplasma in tissue-culture cells. It has since been reported that growth of mycoplasmas occurs: (*a*) Without production of visible cytopathic effect (c.p.e.)—(Robinson, Wichelhausen & Roizman, 1956; Rothblat, 1960; Pollock, Kenny & Syverton, 1960; Carski & Shepard, 1961), although it may result in depression of the growth of the tissue-culture cells (Kenny & Pollock, 1963); or (*b*) with the production of a visible c.p.e. of varying type and degree (Hayflick & Stinebring, 1960; Nelson, 1960; Casterjon-Diez, Fisher & Fisher, 1963; Rovozzo, Luginbuhl & Helmboldt, 1963; Grumbles, Hall & Cummings, 1964; Butler & Leach, 1964). During the course of investigation of outbreaks of bovine infertility strain M 120 of *Mycoplasma bovigenitalium* was isolated from vaginal swabs obtained from a heifer showing lesions of granular vulvovaginitis (Afshar, Stuart & Huck, 1966). This paper gives a detailed account of the growth and cytopathic effect of this strain in tissue-culture systems. The effect of concurrent infection of the tissue culture with a strain of I.B.R. virus and mycoplasmas is also described.

METHODS

Organism. The M 120 strain of *Mycoplasma bovigenitalium* (Afshar *et al.* 1966) was used throughout the experiments, the strain had previously been passaged twice in calf-kidney cell cultures (M 120 CK2). A stock suspension of M 120 was prepared by collecting the supernatant after freezing and thawing and light centrifugation (174*g* for 5 min.). The stock suspension contained $10^{3.3}$–$10^{6.7}$ organisms/ml.

Preparation of mycoplasma 'toxins'

(*a*) M 120CK2 was filtrated through a gradocol membrane (19 μ a.p.d.) using the technique described by Elford (1931).

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(*b*) M 120CK2 was centrifuged at 120,000*g* for 2 hr. The supernatant was collected and treated with Kanamycin as described by Pollock *et al.* (1960). Both the filtrate and the supernatant preparations were shown to be free from mycoplasmas by culturing on Edward agar medium (Edward & Fitzgerald, 1952).

Cell cultures. Primary calf-kidney and pig-kidney cell monolayers were prepared in 2 oz. medical flats by the trypsinization method of Dulbecco & Vogt (1954) modified by Youngner (1954). The cells were grown in Hanks saline containing 10 % (v/v) inactivated calf serum, 0·5 % (w/v) lactalbumin hydrolysate, 0·01 % (w/v) yeast extract to which had been added 100 units penicillin, 100 μg. streptomycin, 100 units polymixin B and 25 units mycostatin per ml. Primary monkey-kidney cell monolayers were obtained from the Wellcome Research Laboratories, Beckenham, Kent. Secondary calf-kidney cell monolayers were prepared on glass coverslips ($\frac{1}{4} \times \frac{7}{8}$ in.). All cultures were shown to be free from any contaminant mycoplasmas by culturing the fluids on Edward agar medium.

Infection of cell cultures. The primary calf-, pig-, and monkey-kidney cell monolayers were washed with phosphate buffered saline and inoculated with 0·5 ml. of a stock suspension of M 120 grown in Edward liquid medium. The organism was allowed to adsorb on to the cells by incubation of the cultures at 37° for 30 min. The cultures were then overlaid with Earle saline containing 2 % (v/v) inactivated horse serum, 0·5 % (w/v) yeast extract and 100 units penicillin per ml. (E.Y.L.). Cultures were examined daily for c.p.e. and harvested 10–14 days after inoculation. For cytological examination secondary calf-kidney cell monolayers were inoculated with 0·1, 0·2, 0·5 or 1·2 ml. of the stock suspension of M120CK2. Following adsorption the cultures were overlaid with E.Y.L. to give a final volume of 1·2 ml. per culture. Cultures were examined daily and the coverslips removed at varying intervals, fixed in Bouin fluid and stained with haematoxylin and eosin. Calf-kidney cell monolayers grown on coverslips were also inoculated with similar volumes of the 'toxins' preparations of M120CK2.

Growth-curve studies. Primary calf-kidney cell monolayers grown in Thompson bottles (each consisting of approximately $2{\cdot}5 \times 10^7$ cells) were inoculated with 10 ml. of the stock suspension of M120CK2. This gave a mycoplasma:cell ratio in one experiment of 1:8890 and in two further experiments of 1:56. After adsorption each culture was overlaid with 170 ml. of E.Y.L. In order to study the growth of the mycoplasma in tissue-culture medium alone, similar bottles, each containing 170 ml. of E.Y.L. without penicillin were inoculated with 1·0 ml. of the stock suspension of M120CK2. All bottles were incubated at 37° and at varying intervals 0·5 ml. samples of the supernatants were withdrawn, diluted in buffered saline and titrated by the drop method described by Miles & Misra (1938) using Edward agar medium. After 48 hr incubation at 37° in a moist atmosphere the colonies were counted and the number of organisms per 1·0 ml. of the supernatant was calculated.

The growth of I.B.R. virus in calf-kidney cell cultures previously infected with mycoplasma.

A primary calf-kidney cell monolayer grown in a Thompson bottle was infected with 1·0 ml. of the stock suspension of M120CK2 as described above. Forty hr later, the supernatant was removed and the cell monolayer was washed with buffered saline. Control (mycoplasma-free) and infected cultures were inoculated with 1·0 ml. of the Oxford strain of I.B.R. virus (Dawson *et al.* 1962). The virus suspension had a titre of $10^{5{\cdot}5}$ tissue culture infecting doses (t.c.i.d. 50) per ml. Cultures were adsorbed and overlaid as described above. At varying intervals 0·5 ml. of the supernatant from each

bottle was removed and assayed for virus content as described by Dawson *et al.* (1962). Samples from the cultures infected with mycoplasmas were first treated with Kanamycin (see above) in order to inhibit further growth of the mycoplasmas. The end-points of virus titration were calculated using the method described by Kärber (1931).

RESULTS

Cytopathology of Mycoplasma bovigenitalium and mycoplasma 'toxins'. Calf-kidney cell monolayers grown on coverslips infected with 0·1 ml. of M120CK2 did not show any changes until 48 hr after infection when the cytoplasm of the cells became granular. The granules were perinuclear and their number had increased by 52 hr (Pl. 1, fig. 2). Compared with the uninfected controls (Pl. 1, fig. 1) there was a marked enlargement of the cells which became accentuated by 148 hr (Pl. 1, fig. 3) when the surviving cells were about 6 times as large as the controls. Most of the infected cells had become detached from the glass by this time. However, the detachment of the cell sheet was not complete even at 240 hr after inoculation when about 20–30 % of the cells, all showing granulation of the cytoplasm, still remained attached on to the glass surface. Examination of the fixed and stained preparations 72 hr after infection showed numerous eosinophilic intracytoplasmic inclusions, each one surrounded by a halo (Pl. 2, fig. 5). The inclusions were irregular in size and shape with a sponge-like structure. The stained control calf-kidney cell monolayers (Pl. 2, fig. 4) showed very few cells bearing homogeneous cytoplasmic inclusions after an incubation period of 148 hr. The development of the complete cycle of c.p.e., i.e. granulation, enlargement and detachment of cells, was found to be related to the volume of inoculum as shown in Table 1.

Table 1. *Cytopathology of Mycoplasma bovigenitalium and mycoplasma 'toxins' in calf-kidney cell cultures grown on coverslips*

Time after inoculation (hr)	*M. bovigenitalium* (10^6 org./ml.) (ml.)				'Toxin' preparations (ml.)			
	0·1	0·2	0·5	1·2	0·1	0·2	0·5	1·2
24	—	G.E.	G.E.	G.E.D.	—	E	G.E.	G.E.D.
48	G.E.	G.E.	G.E.D.	+	—	E	G.E.	+
52	G.E.	G.E.D.	+	+	—	E	G.E.	+
72	G.E.D.	+	+	+	—	E	G.E.	+
120	+	+	+	+	—	E	G.E.	+
148	+	+	+	+	—	E	G.E.	+
192	+	+	+	+	—	E	G.E.	+
240	+	+	+	+	—	E	G.E.	+

G = granulation; E = enlargement; D = detachment; + = complete cytopathic effect; — = no cytopathic effect.

Complete c.p.e. occurred 24 hr after inoculation of calf-kidney cell cultures only when a large volume (1·2 ml.) of mycoplasma 'toxins' was used (Pl. 2, fig. 6). The c.p.e. was incomplete when smaller volumes of 'toxins' were used (Table 1). When showing c.p.e. these cultures were found to be free of any mycoplasma by the culture of the supernatants on Edward medium. The addition of arginine (4·0 m./ml.) did not prevent the appearance of c.p.e. in cultures inoculated with either M120CK2 or mycoplasma 'toxins'.

The changes in infected pig- and monkey-kidney cell cultures produced by M120 CK2 were similar to those described for calf-kidney cells, except that the c.p.e. developed more rapidly in monkey-kidney cell cultures and led to total destruction of the cells within 120 hrs.

Growth curve of Mycoplasma bovigenitalium in calf-kidney cell culture and tissue culture medium

The growth curves of *Mycoplasma bovigenitalium*, strain M120CK2, in the supernatant of calf-kidney cell cultures obtained by the colony count method (Fig. 1) resembled a bacterial growth curve. The doubling time during the logarithmic phase

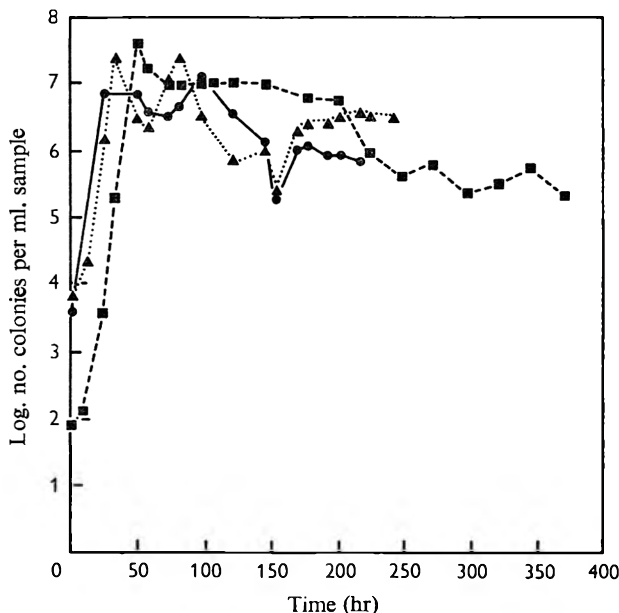

Fig. 1. The growth of *Mycoplasma bovigenitalium* in calf-kidney cell cultures. All cultures measured by colony count of viable particles in the supernatants, ●, ■, ▲ are 3 experiments.

was about 2·2 hr. The maximum titres were reached 32–48 hr after inoculation by which time the cell-culture medium had become turbid. The turbidity, due both to the detachment of the cells and to the increase in the number of organisms, increased during the stationary and the decline phase. During the period of the experiments the pH, estimated using standard paper indicators, varied from 7·6–6·8.

In cell-free medium the growth of mycoplasma was irregular—frequently no viable organisms could be detected in undiluted samples (Table 2). However, in all the experiments the number of organisms increased, ranging from 63 to 158,500-fold. In one experiment (Table 2, Expt. 1) the growth in cell-free medium was similar to that in cell cultures. The pH of the medium increased from 7·6 to 8·8 during the course of the experiments.

Growth of I.B.R. virus in mycoplasma-infected calf-kidney cell cultures. In cultures previously infected with *Mycoplasma bovigenitalium* the growth rate of I.B.R. virus was initially delayed although the final titre of virus produced did not differ significantly

from that produced in mycoplasma-free cultures (Table 3). A delay of about 25 hr in the appearance of c.p.e. in the cultures infected with mycoplasma was also noted. Examination of stained cultures showed that the number of I.B.R. virus intranuclear inclusions was decreased in mycoplasma-infected cultures and they did not appear until somewhat later after inoculation. In cultures infected with both the mycoplasma and I.B.R. virus there were more enlarged cells with 3–6 nuclei per cell than in control virus cultures.

Table 2. *Growth of Mycoplasma bovigenitalium in cell-free tissue-culture medium*

Time (hr)	Expt. 1	Expt. 2	Expt. 3	Expt. 4	Expt. 5
	Log. colony count per ml.				
0	3·7	1·8	2·4	1·7	0
8	3·6	1·8	3·0	0	0
24	6·8	0	2·8	3·0	2·3
32	8·0	0	0	3·9	n.t.
48	6·8	0	2·0	2·7	3·1
56	6·4	0	2·1	4·8	n.t.
72	5·9	0	3·0	4·9	2·0
80	6·1	0	0	4·8	3·1
96	5·4	0	2·8	n.t.	3·7
120	5·1	4·6	0	n.t.	4·3
144	4·7	7·3	0	n.t.	5·9
168	5·3	7·0	0	n.t.	5·65
176	5·9	7·1	3·0	n.t.	n.t.
192	5·9	n.t.	4·9	n.t.	5·4
200	6·0	5·8	4·9	n.t.	n.t.
216	5·6	n.t.	4·9	n.t.	5·6
240	n.t.	4·7	4·5	n.t.	5·4

n.t. = not tested.

Table 3. *Growth of I.B.R. virus in mycoplasma-infected and normal calf-kidney cell cultures*

Time (hr)	Infected	Normal
	Log. tissue culture infecting dose 50 I.B.R. virus per ml.	
0	3·5	3·25
8	3·25	1·75
24	3·25	4·75*
34	3·25	5·5
49	4·5*	5·2
60	4·5	5·5
72	5·0	5·5
80	5·5	5·75

* Appearance of I.B.R. virus c.p.e.

DISCUSSION

The only previously recorded attempt on the growth of *Mycoplasma bovigenitalium* in cell cultures is that of Butler & Leach (1964) who reported that this organism failed to grow in a human cell line (HEp. 2). It has been shown that a related strain, M 120, grew and produced a marked c.p.e. in primary calf-, pig-, and monkey-kidney cell

cultures. The c.p.e. was characterized by enlargement of the cells, appearance of intracytoplasmic inclusions and partial destruction of the cell monolayers. These characteristics resemble those described for other types of mycoplasma. The increase in the granularity of cell monolayers and appearance of the cytoplasmic inclusion bodies have been observed by Hayflick & Stinebring (1955, 1960), Shepard (1958) and Rovozzo *et al.* (1963). Based on their histological observations, these workers suggested that the inclusions and granulations were intracellular forms of the organism. Barile, Malizia & Riggs (1962) and Clyde (1963) using fluorescent antibody techniques showed that the cytoplasmic granulations and inclusions, produced by contaminant mycoplasmas or Eaton agents, were aggregates of the organisms. Similar intracytoplasmic inclusions to those described by Shepard (1958) have been observed both in cells infected with *M. bovigenitalium* and the mycoplasma 'toxins', suggesting that from the appearance of cytoplasmic inclusions or granules the growth of mycoplasmas in cell cultures does not necessarily imply intracellular existence. From the cytological and growth studies of *M. bovigenitalium*, M 120 strain, both in calf-kidney cell cultures and cell-free tissue-culture medium, it is reasonable to assume that this organism grew extracellularly and caused c.p.e. when sufficient toxic substances had been produced or when the medium had been depleted of substances necessary for tissue-culture cell maintenance. These suggestions are supported by Powelson (1961), Kenny & Pollock (1963) and Rouse, Bonifas & Schlesinger (1963) who showed that the multiplication of contaminant mycoplasmas in tissue culture medium depleted the medium of L-arginine which subsequently resulted in changes in the tissue-culture cells. Kraemer (1964) showed that the depletion of L-arginine was in fact due to the production of the mycoplasma 'toxins'. Whether or not the toxin produced results in enzymic degradation of L-arginine is not yet clear. In contrast to Kraemer's findings that the addition of L-arginine to the cell-culture medium prevented the cytotoxic effects, the c.p.e. caused by *M. bovigenitalium* or the 'toxins' was not prevented by the addition of 4 times the normal concentration of L-arginine to the medium.

The growth of *Mycoplasma bovigenitalium* in cell-free tissue-culture medium was irregular compared to the growth in the presence of calf-kidney cells, suggesting that the cells or cell residues were utilized by mycoplasmas and resulted in more regular growth. The regular growth in the presence of cells is comparable with the growth of other mycoplasmas studied in synthetic media (Kelton, 1960; Butler & Knight, 1960). The growth of mycoplasmas in cell-free tissue-culture medium has been reported by Nelson (1960) and Fabricant, Fabricant & van Demark (1964). Fabricant *et al.* (1964) found that the addition of ribonucleotides and deoxyribonucleotides to '199' medium improved the growth of *M. bovigenitalium*. This supports our suggestion that the regular growth of a related strain (M 120) in calf-kidney cell cultures was due to the utilization of cell residues.

According to Rouse *et al.* (1963) and Butler & Leach (1964) the growth of adenovirus and myxovirus in cell cultures contaminated with mycoplasmas was inhibited. Rouse and his colleagues demonstrated that inhibition of virus multiplication was directly caused by the depletion of arginine from the medium by the mycoplasmas, and the addition of arginine increased the virus yield. According to Schulze & Schlesinger (1963) the growth of the arbovirus—Dengue-type 2—was not affected in cell cultures contaminated with mycoplasma. It has been shown that growth of I.B.R. virus, a herpesvirus (Armstrong, Pereira & Andrews, 1961), proceeded initially at a slower rate

and that the onset of the visible c.p.e. was delayed in the presence of concurrent infection with *Mycoplasma bovigenitalium*. This finding is of particular interest as I.B.R. virus has been shown to be identical to infectious pustular vulvovaginitis (I.P.V.) virus (McKercher, Straub, Saito & Wada, 1959; Gillespie, McEntee, Kendrick & Wagner, 1959) which is a natural pathogen of the genitalia of cattle as is *M. bovigenitalium* (Edward & Fitzgerald, 1952). Kendrick, Gillespie & McEntee (1958) showed that I.P.V. virus caused lesions in a similar situation to that described for *M. bovigenitalium* (Afshar *et al.* 1966).

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Uninfected calf-kidney cell monolayer, unfixed. ×140.

Fig. 2. *Mycoplasma bovigenitalium*-infected calf-kidney cell monolayer (0·1 ml.), unfixed, 52 hr after infection, showing perinuclear granules. ×140.

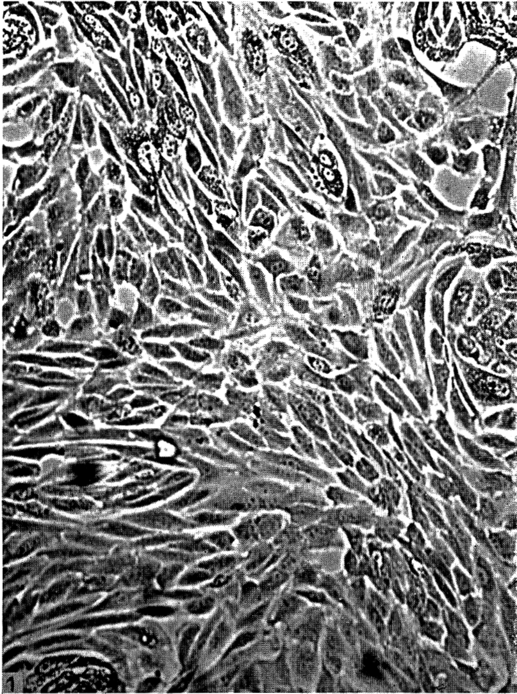
Fig. 3. *M. bovigenitalium*-infected calf-kidney cell monolayer (0·1 ml.), unfixed, 148 hr after infection, showing enlarged cells. ×140.

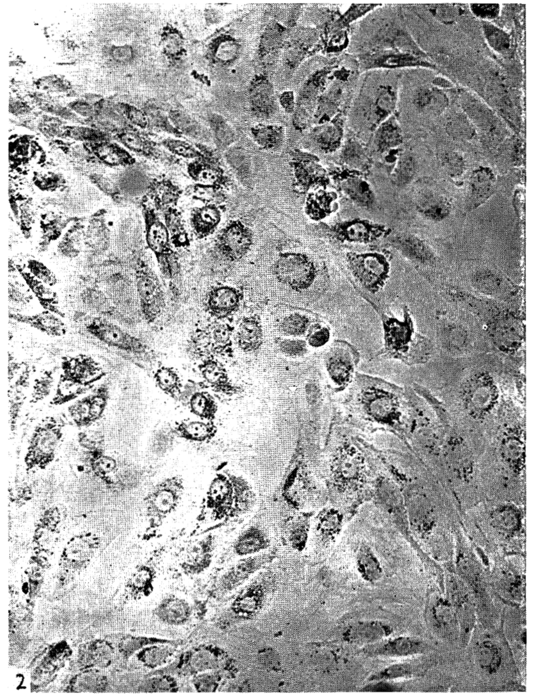
PLATE 2

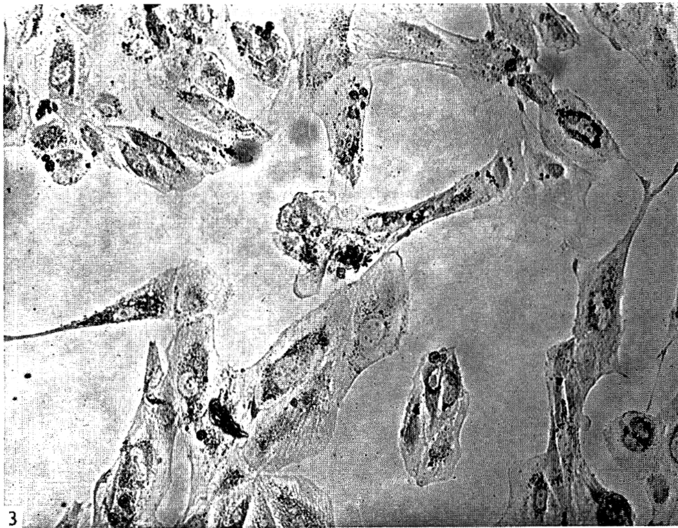
Fig. 4. Uninfected calf-kidney cell monolayer, fixed, and stained with haematoxylin and eosin. ×552.

Fig. 5. *Mycoplasma bovigenitalium*-infected calf-kidney cell monolayer, 72 hr after infection, fixed and stained with haematoxylin and eosin, showing cytoplasmic inclusions surrounded by haloes. ×552.

Fig. 6. Calf-kidney cell monolayer inoculated with mycoplasma 'toxins', unfixed, 24 hr after inoculation. ×140.







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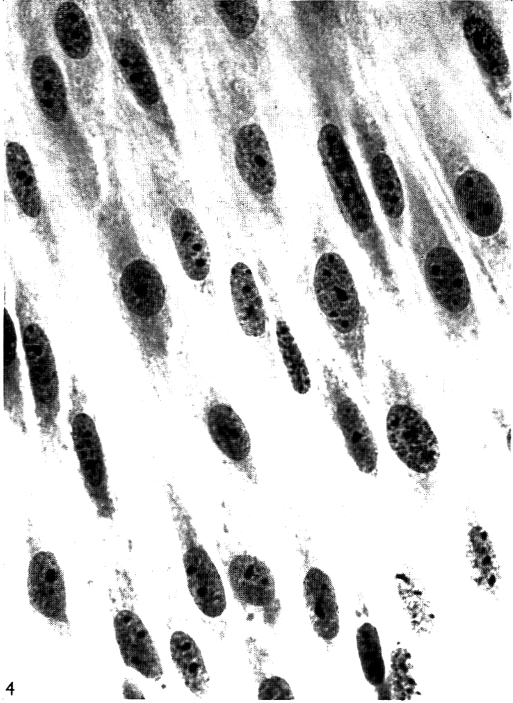

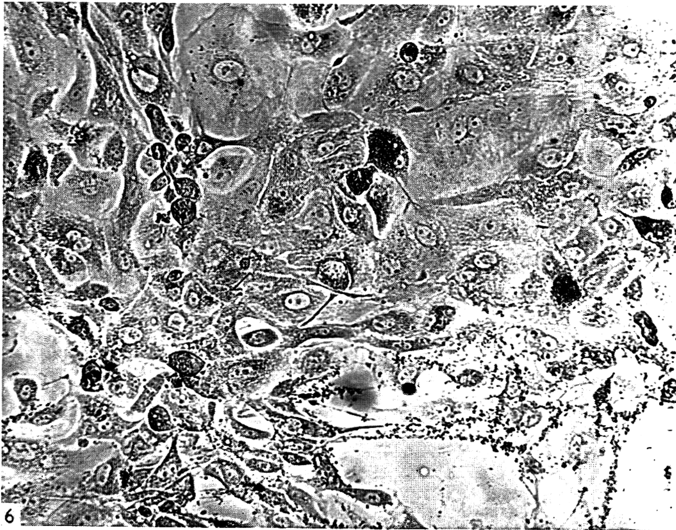

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Inhibition of Growth and Nucleic Acid Synthesis in Iron-Deficient *Mycobacterium smegmatis*

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SUMMARY

Comparative studies of growth, yield of organism and synthesis of nucleic acids were made on a strain of *Mycobacterium smegmatis* grown with and without sufficient iron. Although the bacteria grew exponentially at the same rate in the iron-deficient medium as in the iron-replete medium, the content of RNA and DNA was lower in the iron-deficient bacteria. In the iron-deficient cultures, growth ceased abruptly after an exponential phase characterized by an inhibition of DNA synthesis. The pH value of the medium became increasingly acidic as iron-deficient growth proceeded.

INTRODUCTION

The need for iron as an essential element for the production of enzymes involved in the cytochrome system for production of high-energy phosphate is well established (Theodore & Schade, 1964). Poor growth results with many bacteria when the media are depleted of iron, and the growth obtained is often proportional to the amount of the iron available in the medium. The response of DNA and RNA synthesis to changes in growth rate, shift-up and shift-down, induced by medium changes have been studied in detail for several organisms (Herbert, 1961; Schaechter, Maaløe & Kjeldgaard, 1958; Schaechter, 1961; Neidhart & Fraenkel, 1961; Neidhart & Magasanik, 1960). Growth-rate changes, induced by carbon and/or nitrogen-source variations, result in predictable effects on RNA and DNA synthesis. As the growth rate decreases, the rate of synthesis of RNA decreases. This slowing of RNA synthesis precedes the slowing of DNA synthesis. As growth rate increases (as in shift-up) RNA responds first and DNA later. Such results led Herbert (1961) to formulate rules describing the shifts in nucleic acid content of bacteria. Thus the RNA and DNA content of *Aerobacter aerogenes* and *Bacillus megatarium*, to mention only two (Herbert, 1961), did vary according to these simple rules, under variations of supplies of carbon source and nitrogen source. These rules have not been so exclusively tested with growth changes induced by variations in the concentrations of nutrient minerals. The present work describes the growth and content of RNA and DNA of *Mycobacterium smegmatis* grown in submerged cultures in chemically defined media. Iron-replete cultures were expected to show the usual pattern of nucleic fluctuations as described by Herbert (1961) for other bacteria, since, in the present author's view, the sequence of growth of bacteria in a closed system can be viewed as a gradual 'shift-down'. Certain features of the growth of *M. smegmatis* were found to be typical, but the DNA fluctuation was

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not. It was not expected that the content of organisms grown in the iron-replete medium would differ from the content of the organisms grown in the iron-deficient medium during the exponential phase, since equality of growth rate usually results in identity of composition (Schaechter, 1961). Differences were found in the composition of the bacteria mass according to the concentration of added iron. Previous studies of the composition of *M. smegmatis* which correlated growth, cell content and mineral supply, were confined to surface cultures (Winder & O'Hara, 1962). Such cultures have inherent drawbacks, particularly in that two types of bacterial populations exist: one on the surface of the pellicle, and a population more or less submerged in the medium, below the pellicle.

METHODS

Organism. The strain of *Mycobacterium smegmatis* used was obtained from the Medical Research Council of Ireland's Laboratories, Trinity College, Dublin. It is very similar to NCTC 8152, and is the strain used by Winder & O'Hara (1962).

Media. The bacteria were grown in submerged culture using Proskauer & Beck (Winder & O'Hara, 1962) medium. It had the following composition: asparagine, 5·0 g.; magnesium citrate, 1·5 g.; KH_2PO_4, 5·0 g.; glycerol, 20·0 ml.; glass-distilled water to 1 l.; adjusted to pH 7·0 by addition of 5 N-KOH solution before adding the magnesium citrate. The chemicals used were all of analytical reagent grade, except magnesium citrate, which was a British Drug Houses Limited product.

The medium was depleted of trace metals by autoclaving with alumina as described by Winder & O'Hara (1962) except that alumina was added to the medium to give a 2 % (w/v) suspension. A chromatographic grade of alumina was used (May and Baker Ltd., Dagenham, Essex). After filtration to remove the alumina, iron-replete media was prepared by adding iron, as a solution of $FeSO_4 . 7H_2O$, to give a concentration of 2·0 μg. added Fe^{2+}/ml. Zinc was added as a solution of $ZnSO_4 . 7H_2O$, to give a concentration of 0·4 μg. added Zn^{2+}/ml. to iron-replete medium, and to iron-deficient medium (medium with no added iron).

The media were then dispensed in 80 ml. or 160 ml. volumes into 250 ml. conical Pyrex flasks.

The following procedure was applied to flasks, pipettes, Pasteur pipettes, ball mills and beads, in order to remove traces of iron and zinc from their surfaces. Glassware was soaked for 2 days in ethanolic KOH (5 %, w/v), then rinsed with tap water, soaked in nitric acid (a 50 % (v/v) solution of conc. HNO_3 sp.gr. 1·42, in water), rinsed with tap water (2 l./250 ml. flask), and washed four times with glass distilled water. All glassware was dried in air before use.

Pipettes and other glassware were sterilized by heating for 2 hr at 160°.

Preparation of inocula. Bacteria from a 3-day surface culture of *Mycobacterium smegmatis* grown at 37° were suspended in fresh media and lightly ground in a ball mill. Portions of the suspension were pipetted to 250 ml. flasks containing media (160 ml. or 80 ml.) to give, approximately the equivalent of $1·5 \times 10^{-4}$ mg. dry wt. bacteria/ml. in the resulting suspension.

Growth and harvesting of bacteria. Inoculated flasks were placed in a gyratory incubator-shaker (model G 25; New Brunswick Scientific Co. Inc., New Brunswick, N.J., U.S.A.) at 37° and shaken at 220 rev./min. (with an amplitude of 1 in.). After incubation the contents of a flask were decanted into 100 ml. nylon centrifuge tubes

and centrifuged (2500*g*) for 10–15 min. at 0°. The supernatant fluid was retained for pH determination, and the deposit resuspended in up to 10 ml. ice-cold distilled water, pooled with similar deposits when necessary, and then centrifuged (2500*g*) again in 16 × 125 mm. tared test-tubes. The supernatant fluid was discarded. Traces of supernatant fluid were removed with a fine pipette. The centrifuged deposit residue was freeze-dried at −30° and dried over P_2O_5 for 2 days. Where necessary the contents of several flasks were pooled before drying. The weights of the centrifuged deposits were determined by difference.

Extraction procedures. Amounts from 50 to 300 mg. of freeze-dried organism were extracted. To the bacterial mass in 16 × 25 mm. test-tubes, 0·5 ml. water was added, and then the mass was treated with absolute ethanol (5 ml. at 18° for 30 min.), and then successively extracted (3 × 5 to 10 ml.) with ethanol ether (3+1, v/v, 60°). These extracts and the ethanol extract were pooled for phospholipid determination. After each extraction the suspension was centrifuged at 2000*g* for 5 min. at 60°.

The residue was then extracted for the acid soluble pool by the following procedure. The mass was agitated with 10 ml. 5 % (w/v) trichloroacetic acid (TCA) for 3 hr at 0°. The suspension was then centrifuged (2000*g*) for 5 min. at 0°. The supernatant fluid was retained, and the residue again extracted with fresh TCA (5 ml. for 30 min.) as before, and centrifuged as before. The supernatant fluid was pooled with the first acid extract. The deposit was then washed with fresh TCA (5 ml., 0°) centrifuged as before, and the washings added to the pooled supernatant fluids. Finally the extracted deposit was washed with 5 ml. water at 0°, centrifuged, and the supernatant fluid added to the pooled TCA extracts. These extracts were pooled for nicotinamide moiety determination, and to determine the extinction of the 260 mμ absorbing material spectrophotometrically.

The residues were then treated with N-KOH (10 ml. for 18 hr at 25°) and then neutralized with 60 % (w/v) $HClO_4$. Further perchloric acid was then added to the samples to give a 3 % solution of the acid, in which the residue was suspended and agitated at 0° for 3 hr, and then centrifuged at 2500*g* for 15 min at 0°. The deposit was then treated with 3 % (w/v) perchloric acid (2 × 5 ml. at 0°). The supernatant fluids and washings were pooled and diluted with 3 % $HClO_4$ to a fixed volume (20, 25 ml. depending on the experiment) for determination of RNA.

The residues after perchloric acid extraction were extracted with TCA (10 ml. of 5 % (w/v) for 15 min. at 90°) stood at 0°, and then centrifuged. The residues were then washed with two portions (5–7 ml.) of 5 % (w/v) TCA at 18°, resuspended, and centrifuged; the supernatant fluids were then pooled and brought up to a fixed volume (20 or 25 ml.) with TCA (5 %, w/v) for determination of DNA.

In the extraction procedure described above, centrifugation was done at 0°, for 5–10 min. at 2000*g* unless otherwise stated. During the extractions the deposits were suspended and agitated by stirring with fine glass rods, and supernatant fluids removed by decantation and finely tapered Pasteur pipettes.

Determination of nucleic acids. The solutions containing the nucleic acids were determined for RNA and DNA spectrophotometrically by measuring their extinctions (1 cm.) at 260 mμ and 269·5 mμ. Concentrations are expressed in atoms of phosphorus by using the atomic extraction coefficient given by Logan, Mannel & Rossiter (1952).

RESULTS

Growth of Mycobacterium smegmatis

The exponential doubling time of *Mycobacterium smegmatis* in these cultures was found to be 7·0 ± 0·5 hr. Growth departed from the exponential at about 7 generation times after inoculation both in iron-replete and in iron-deficient media. Growth in the iron-replete medium continued to increase for a further three generation times; in the iron-deficient medium growth was nearly stationary after the exponential period. Whereas the slowing down in growth rate in the iron-replete medium was gradual, there appeared to be an abrupt transition from exponential to stationary growth in the iron-deficient medium (Fig. 1).

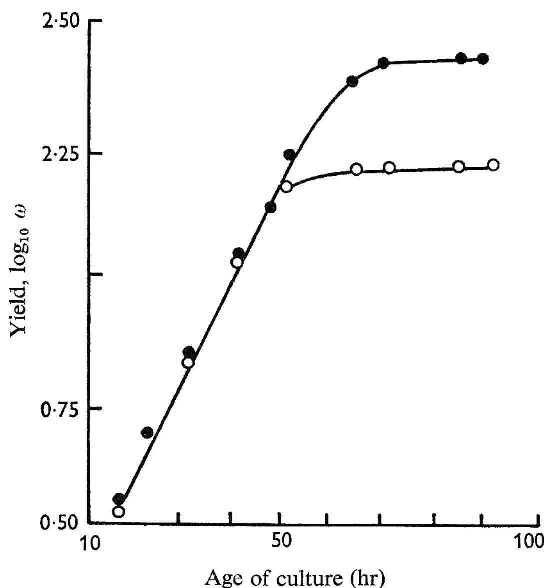

Fig. 1. Growth of *Mycobacterium smegmatis* in chemically defined glycerol asparagine medium at 37° with different concentration levels of added iron. Iron-replete organisms (●) were grown in media containing 2·0 μg. added Fe^{2+}/ml. and 0·4 μg. added Zn^{2+}/ml. Iron-deficient organisms (○) were grown in media containing 0·4 μg. Zn^{2+}/ml. but no added iron. The yield is expressed as W = equiv. mg. dry wt. organism from 80 ml. culture.

The stationary phase in the iron-replete cultures was approached gradually (Fig. 1; Table 1) and was achieved about 7·5 generation times after inoculation.

The gradual slowing of growth in the iron-replete cultures is typical of growth obtained when several nutrients are exhausted (Herbert, 1961), in the present case exhaustion of nitrogen and carbon sources was probably the cause (Winder & O'Hara, 1962). However, the yields of organism obtained in cultures with sufficient iron varied, although the pattern of growth and the timing of the onset of the growth phases was constant. The yield for cultures grown in 250 ml. flasks containing 160 ml. medium was less than when 80 ml. was used. The limiting factor here was the rate of oxygen diffusion into the culture, since with a larger volume of medium the surface area of medium in contact with the atmosphere, per ml. of medium, was less.

The abrupt transition from exponential growth to the stationary phase in iron-deficient culture is indicative of the exhaustion of a single nutrient (Herbert, 1961), in this case iron.

The stationary phase resulting from iron-deficiency can persist for at least 2 days without lysis of the bacteria.

Table 1. *Yields of organism and nucleic acid content of Mycobacterium smegmatis*

Mycobacterium smegmatis was grown in 80 ml. glycerol asparagine medium at 37°. The iron-replete medium contained 0·4 μg Zn^{2+}/ml. and 2·0 μg. added Fe^{2+}/ml. The iron-deficient medium contained 0·4 μg. added Zn^{2+}/ml. but no added iron. The bacteria were harvested at the times indicated, freeze-dried, weighed, and their nucleic acids extracted.

Iron-replete medium				Iron-deficient medium		
Yield (mg. dry wt. bacteria 80 ml. culture)	μg. P/mg dry wt. bacteria.		Age of culture (hr)	Yield (mg. dry wt. bacteria/ 80 ml. culture)	μg. P/mg dry wt. bacteria.	
	P-DNA*	P-RNA*			P-DNA*	P-RNA*
192	2·70	4·95	45	184	1·50	5·45
286	2·55	5·05	50	229	1·40	4·00
285	2·55	5·27	53	240	1·30	4·50
348	2·55	4·80	56	248	1·20	4·25
442	2·70	4·87	70	266	1·10	3·84
498	3·00	4·72	75	269	1·05	3·60
562	2·85	4·40	98	269	1·00	3·60

* Concentrations of RNA, DNA as atoms of phospherus (Logan, Mannel & Rossiter)

Synthesis of RNA in post-exponential growth of Mycobacterium smegmatis

The rate of synthesis of RNA in iron-replete cultures of *Mycobacterium smegmatis* slowed to a greater extent than did the growth rate, as shown by the decrease in the values of P-RNA/unit dry wt. organism. This result is typical of bacteria when undergoing a 'shift-down' in growth rate (Herbert, 1961). This effect was shown by *M. smegmatis* grown in iron-replete and iron-deficient media (Table 1). However, even during inhibition of growth by iron deficiency the content of P-RNA/dry wt. organism continued to decrease.

Synthesis of DNA during post-exponential growth of Mycobacterium smegmatis

DNA synthesis is less sensitive to growth-rate changes than RNA synthesis (Herbert, 1961). The content of P-DNA of *Mycobacterium smegmatis* grown in iron-replete media appeared to increase during the transition from declining phase to stationary phase. In 'shift-down' experiments the content of DNA of bacteria increases (Herbert, 1961). In the stationary phase of *M. smegmatis* grown in iron-deficient medium the content of DNA continued to decrease and reached a constant value (Table 1). Thus whereas a shift-down resulting from carbon and nitrogen deficiency led to the usual DNA response in *M. smegmatis*, a shift-down resulting from an iron deficiency led to an abnormal slowing in DNA synthesis.

Synthesis of RNA during exponential growth of Mycobacterium smegmatis

There appeared in the early exponential phase of *Mycobacterium smegmatis* growing in iron-replete medium to be a higher rate of synthesis of RNA than of cell mass. This was shown by the increase in content of RNA (Table 2) in these cultures. Before the declining phase was reached in these cultures, the synthesis of RNA accelerated, and fell behind the rate of increase of cell mass, leading to a decrease in RNA content. This decline in the rate of RNA synthesis before a declining growth rate is well known (Herbert, 1961). In cultures of *M. smegmatis* grown in iron-deficient media, a similar decline of RNA synthesis before a decline of growth rate was also found (Table 2). According to Herbert (1961), an identical growth rate should lead to a similarity of chemical composition in the bacteria. However, this was not found here with *M. smegmatis*, where, although an exponential doubling rate of $7 \cdot 0 \pm 0 \cdot 5$ hr was found both in iron-replete and in iron-deficient cultures, the content of RNA was lower in the latter (Table 2).

Table 2. *Yield of organism and nucleic acid content of Mycobacterium smegmatis during exponential growth*

Mycobacterium smegmatis was grown in the glycerol asparagine medium at 37°. The iron-replete medium contained 0·4 μg. added Zn^{2+}/ml., and 2·0 μg. added Fe^{2+}/ml. The iron-deficient medium contained 0·4 μg. Zn^{2+}/ml., but no added iron. The bacteria were harvested at the times indicated, freeze-dried, weighed, and their nuclei acids extracted.

	Iron-replete media			Iron-deficient media		
Age of culture (hr)	Yield mg. dry wt. bacteria/ 80 ml. culture	μg.P/mg. dry wt. bacteria: P-DNA	P-RNA	Yield mg. dry wt. bacteria/ 80 ml. culture	μg.P/mg. dry wt. bacteria: P-DNA	P-RNA
	Cultures in 160 ml. medium					
18	3·5	2·9	6·9	4·4	3·8	6·9
23	7·6	2·9	7·8	7·4	3·2	6·9
28	16·8	3·5	7·8	15·0	—	—
34	15·3	2·9	7·5	—	—	—
41	35·7	3·2	6·9	42·1	3·0	6·0
48	57·7	2·7	6·1	54·4	2·7	6·3
	Cultures in 80 ml. medium					
21·5	30·6	1·85	4·70	19·7	2·71	6·8
25·5	48·5	2·29	5·80	52·4	2·42	6·12
38·5	139·5	2·33	6·15	138·0	1·12	5·16

Synthesis of DNA during exponential growth of Mycobacterium smegmatis

The content of DNA increased and then decreased as the exponential phase continued in iron-replete cultures of *Mycobacterium smegmatis*. Herbert (1961) generalized that the content of DNA will usually decrease then increase as the decline phase of growth is approached. *M. smegmatis* was not found to follow this pattern (Table 2). In cultures of *M. smegmatis* grown in iron-deficient media, the content of DNA, and therefore the synthesis of DNA, decreased throughout the exponential phase. Previous studies of nucleic acid content during exponential growth (Herbert, 1961) suggested

that the nucleic acid content of the bacteria was dependent only on the growth rate, which was itself defined by the medium. In the case of *M. smegmatis* an identical rate of 7·0 ± 0·5 hr exponential doubling resulted in a different pattern of DNA synthesis, dependent upon the iron content of the culture medium (Table 2).

Inhibition of DNA synthesis during iron-deficient growth of Mycobacterium smegmatis

The content of DNA and RNA in iron-deficient *Mycobacterium smegmatis* decreased during post-exponential growth (Table 1). The results of several experiments suggested that during the exponential phase the synthesis of DNA in iron-deficient cultures lagged behind the rate of synthesis of cell mass. This resulted in a decreasing content μg. DNA/mg. of dry weight of cells of DNA. RNA also decreased in content during the exponential phase (Table 2). In cultures with added iron, however, the synthesis of RNA and of DNA exceeded the rate of synthesis of cell mass; this resulted in an increase of content of RNA and DNA. The rate of synthesis of the RNA and DNA then decreased, but the rate of RNA synthesis decreased before that of DNA (Table 2).

Changes of the pH value of cultures during growth of Mycobacterium smegmatis

The pH value of cultures of *Mycobacterium smegmatis* growing in iron-replete media decreased below pH 7 during the exponential phase, but began to increase again as growth declined. Cultures of iron-deficient *M. smegmatis* became increasingly acid as the exponential phase progressed, and continued to increase in acidity after growth was inhibited (Table 3).

Table 3. *Changes of pH value of medium with growth of Mycobacterium smegmatis in Fe+ and Fe− media*

The bacteria were grown as described in Methods in 80 ml. of medium. At the appropriate times, the bacteria were centrifuged, and the pH value of the supernatant fluid determined at 20°.

Fe-replete. Medium containing 2·0 μg. added Fe^{2+}/ml. and 0·4 μg. added Zn^{2+}/ml	Fe-deficient. Medium containing 0·4 μg. added $Zn.^{2+}$/ml. and no added Fe^{2+}	Age of culture (hr)
7·00	7·00	21·5
6·90	6·90	25·5
6·80	6·86	38·5
6·80	6·91	43·5
—	6·68	50·0
—	6·47	63·0
6·90	6·65	68·0
7·10	6·52	87·0

DISCUSSION

Previous work on nucleic acid synthesis in *Mycobacterium smegmatis* has been confined to the study of nucleic acid content, as measured over several days in surface cultures (Winder & O'Hara, 1962). With their conditions of experiment only post-exponential metabolism could be studied, and therefore the effects of iron deficiency

could only be compared with the effects of carbon + nitrogen deficiency. It was found that, as compared with the DNA and RNA content of carbon-nitrogen-deficient cultures, DNA and RNA synthesis was inhibited in iron-deficient cultures. The present work was designed to investigate the pattern of nucleic acid synthesis in submerged cultures of *M. smegmatis*, and to compare the patterns obtaining in exponential growth in cultures differing only in iron content. From this work it appears that the general patterns cited by Herbert (1961) do not apply to *M. smegmatis* grown with adequate or inadequate iron supplies. During post-exponential growth, synthesis of RNA and DNA in the iron-deficient cultures lagged behind that in the iron-replete cultures (Table 1). This was not entirely unexpected since the yield of organism in iron-deficient medium was less than in the iron-replete medium. However, the synthesis of DNA and RNA in iron-deficient cultures was inhibited to a greater extent than the inhibition of growth, as shown by the decreased contribution of the nucleic acids to the bacterial mass (Table 1).

Tempest, Hunter & Sykes (1965) found that the DNA content of *Aerobacter aerogenes* grown in a chemostat with magnesium limitation varied little, while the RNA content was stoichiometrically related to the concentration of available magnesium; however, no consistent difference in RNA content was observed as between Mg^{2+}-limited cultures and C-limited cultures of *A. aerogenes*. This is to be compared with the results obtained with Fe^{2+}-limited *Mycobacterium smegmatis*, where DNA and RNA synthesis was inhibited, apparently specifically, by deficiency of iron. Growth of *M. smegmatis* limited by carbon + nitrogen deficiency, was associated with the usual pattern of decline of RNA synthesis in the declining growth phase.

Since many oxidation-reduction reactions are dependent on iron-containing haemoproteins, the question arises: is the inhibition of DNA synthesis in iron-deficient *Mycobacterium smegmatis* the result of a breakdown of energy-supplying metabolism? Theodore & Schade (1965) showed that a strain of *Staphylococcus aureus* when grown in iron-deficient media lacked the ability to oxidize lactate, pyruvate and citrate. The carbon source in the media used for the growth of *M. smegmatis* in the present work was mainly glycerol and citrate. If post-glycolytic oxidation is impaired in iron-deficient *M. smegmatis*, a sharp cessation of growth might result, but it is difficult to account for the inhibition of DNA synthesis before growth inhibition on this basis alone. The increase in acidity of the medium in iron deficiency might be the result of excretion of lactate and pyruvate into the medium by bacteria unable to oxidize these compounds further. The increase in acidity of media containing 2·0 μg. added iron/ml. during the exponential phase might be the result of lactate and pyruvate excretion. The decrease in acidity would be explicable in that, since supplies of glycerol decreased as growth continued, aerobic oxidation of lactate and pyruvate would increase.

The effect of iron deficiency on enzyme activity of *Mycobacterium smegmatis* was investigated by Winder, O'Hara & Ratledge (1961). Catalase, peroxidase, succinic dehydrogenase and glycerol dehydrogenase activity was found to decrease in bacteria grown in iron-deficient cultures, in comparison with iron-replete cultures, as the cultures grew older.

Ferrochelatase has been demonstrated in several micro-organisms by Porra & Jones (1963). This enzyme is iron-dependent, and therefore haem production is iron-dependent as well. If the synthesis of haem is inadequate in early exponential cultures of iron-deficient *Mycobacterium smegmatis*, and decreased below the requirements for

oxidation and energy production, a resultant inhibition of growth would be explicable in these terms alone. However, there is no *a priori* reason that the present author can suggest as to why a specific slowing of the production of high-energy phosphate synthesis should specifically inhibit DNA synthesis rather than RNA synthesis. The inhibition of growth of *M. smegmatis* in iron-deficient cultures, if it occurred without the specific inhibition of DNA production described in this paper, would be explicable in terms of inadequate production of high energy phosphate for metabolic purposes.

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Comparison of the Germination and Outgrowth of Spores of *Bacillus cereus* and *Bacillus polymyxa*

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SUMMARY

During germination of *Bacillus cereus* spores, the cortex was lost completely, but with *B. polymyxa* spores there was no apparent alteration in the cortex structure. On the other hand, the quantities of dipicolinic acid, calcium and mucopeptide, measured as hexosamine, released from germinating *B. polymyxa* spores were similar to those released from germinating *B. cereus* spores. It appears that only about 30 % of the mucopeptide of these species is involved in the maintenance of spore dormancy. The solubilization of the spore dipicolinic acid, calcium and mucopeptide during germination only accounted for about half of the loss of dry weight from the spores. During the outgrowth of *B. cereus*, the spore coats dissolved away at one pole and the vegetative form grew out, leaving only fragments of the spore integument free in the medium. *Bacillus polymyxa* grew when the largely unaltered coat and cortex layers of the spore split open at the organism's equator.

INTRODUCTION

The germination of Bacillus spores is characterized by the loss of phase brightness and heat resistance, and a decrease of 30 % in the dry weight with the solubilization from the spore of dipicolinic acid, calcium and hexosamine-containing mucopeptide (Powell & Strange, 1953; Strange & Powell, 1954). During outgrowth, the germinated spore goes through stages of swelling and elongation, the developing vegetative bacillus finally emerging from the discarded outer layers of the spore and going through the first division.

Although there is evidence that the calcium is located in the spore core (Thomas, 1964; Knaysi, 1965), various authors have claimed that the dipicolinic acid, calcium and mucopeptide of the spore are located in the cortex (Mayall & Robinow, 1957; Vinter, 1965; Warth, Ohye & Murrell, 1963) and that the primary event in germination is a dissolution or alteration in the cortex structure (Kawata, Inoue & Takagi, 1963; Mayall & Robinow, 1957). A lytic enzyme capable of hydrolysing the spore mucopeptide has been described (Strange & Dark, 1957) and extracted from *Bacillus cereus* (Strange & Dark, 1957; Gould, 1962; Gould & Hitchins, 1965). Although this lytic enzyme has not been extracted from *B. subtilis*, *B. sphaericus* or *B. coagulans*, there is evidence for its presence in the latter two species. Gould & Hitchins (1965) suggested that a prime event in the germination of all species may be the activation of the lytic enzyme. The outgrowth characteristics of several Bacillus species have been examined (Lamanna, 1940; Gould, 1962). From this work the generalization can be made that species with

large spores, e.g. *B. cereus*, grow out by dissolving away the coat and cortex layers over a large area of the cell surface, whereas with the species with small spores, e.g. *B. subtilis*, the outer layers of the spore split open and the vegetative bacillus emerges, leaving behind largely unaltered fragments of the spore integument. Although no evidence has been obtained of an enzyme which lyses the spore coats, the dissolution of the cortex during outgrowth could be explained by the lytic enzyme, acting over the whole cortex in *B. cereus* but only at a limited number of specific sites in *B. subtilis* (Gould, 1962).

Several studies of thin sections of spores in the electron microscope have been made. Chapman & Zworykin (1957) examined germinating and growing *Bacillus cereus* and showed the gradual dissolution of the spore cortex and coat layers, with fragments of coat still attaching to the vegetative bacilli, even after the first division. Similar results were obtained from the study of *B. anthracis* (Moberley, Shafa & Gerhardt, 1966). In their elegant study of *B. subtilis*, Kawata *et al.* (1963) described the multi-laminate structure of the coat and the undifferentiated appearance of the core in the mature spore. After germination, the cytoplasm of the core, or future vegetative bacillus, developed the characteristic granular appearance with a nuclear area of low electron density. As growth proceeded, the cortex was dissolved away and the vegetative bacillus developed its cell wall and membrane, and eventually grew out by rupturing the spore coat in the equatorial region. Similar findings have recently been reported by Rousseau, Fléchon & Hermier (1966). By staining with lanthanum nitrate, Mayall & Robinow (1957) were able to obtain a very clear picture of cortex structure in spores of *B. megaterium*. During germination the laminated structure loosened and the cortex became spongy. At the same time, the outer layer of the spore coat was thrown off and folded, and the core became granular. During growth the cortex disappeared, the normal cytoplasm, wall and membrane of the vegetative bacillus developed, and the spore coat dissolved away at the polar region to allow the emergence of the vegetative bacillus.

In this study we have examined the characteristic morphological changes during the germination and growth of *Bacillus cereus* and *B. polymyxa* spores, and compared these with the losses of dipicolinic acid, calcium and mucopeptide from the spores during germination, and the re-incorporation of the mucopeptide into the wall of the developing vegetative bacillus.

METHODS

Cultures and media. The two organisms used in this study were laboratory strains of *Bacillus cereus* and *B. polymyxa*. *Bacillus cereus* was the PX strain used by Strange & Dark (1957) for the isolation of the lytic enzyme. These bacteria were grown on the surface of potato glucose agar containing 0·25 % (w/v) glucose, 0·4 % (w/v) yeast extract, 0·4 % (w/v) potato extract and 1·5 % (w/v) agar (pH 7·4). After a large crop of free spores had been produced in 2–5 days at 30°, they were washed off the surface of the agar with ice-cold de-ionized water, washed clean from vegetative forms and agar particles by several centrifugations, heated for 30 min. at 65° and stored at 4° in a suspension containing 10^8–10^9 spores/ml. Before each experiment the spores were heat activated by heating at 65° for 30 min.

The germination medium for *Bacillus cereus* was 10 ml. spore suspension, 60 ml. 0·15 M-phosphate buffer (pH 7·6), 15 ml. of 10 mg. alanine/ml. solution and 15 ml.

of 10 mg. inosine/ml. solution. The germination medium for *B. polymyxa* was 10 ml. spore suspension, 240 ml. 0·15 M-phosphate buffer (pH 7·6) and 50 ml. of 10 mg. alanine/ml. solution. Incubation was for 1–2 hr at 30°, until 95–100% germination had taken place, as assayed by loss of phase brightness. The extent of germination was verified by colony counts after heating to 65° for 30 min. to kill the germinated organisms. Control samples were obtained by making the above dilutions in de-ionized water, rather than in germination media. After germination the organisms were washed twice in ice-cold de-ionized water and resuspended to 30 ml.

Germination and outgrowth were followed by withdrawing samples at intervals from spore suspensions in a full nutrient medium containing 0·5 % (w/v) glucose, 0·5 % (w/v) peptone, 0·5 % (w/v) $Na_2HPO_4.12H_2O$, 0·5 % (w/v) yeast extract and 0·5 % (w/v) Lab Lemco (pH 7·3). Seven ml. of spore suspension was added to 42 ml. pre-heated medium. Incubation was at 37° with shaking, and 7 ml. samples were pipetted into 25 ml. ice-cold de-ionized water, centrifuged, washed in a further 15 ml. and finally resuspended to 3·5 ml. In this medium the germination of *Bacillus cereus* was so rapid that equivalent dilutions in de-ionized water had to be made in order to get a 'zero time' sample. A portion from each sample after washing was examined under the phase microscope and the stage of germination or outgrowth of the organisms noted.

Chemical analysis. Samples of dormant spores and spores germinated in the germination media were assayed in duplicate for dipicolonic acid (5 ml.), calcium (2 ml.) and, as a measure of mucopeptide, hexosamine (1 ml.). Only hexosamine assays were made on samples from full nutrient media. Dipicolinic acid was assayed by the method of Jansen, Lund & Anderson (1958), after autoclaving the organisms at 121° for 15 min. Calcium was assayed by flame photometry after ashing the organisms at 500° for 16 hr and redissolving the residue in 6 ml. 0·02 N-HCl. Hexosamine was assayed by a modification of the method of Boas (1953). The organisms were hydrolysed for 2 hr at 100° with an equal volume of 12 N-HCl; hydrolysis from 1 to 7 hr solubilized the same amounts of hexosamine. A blank containing water and a set of standards were run with each assay. A second blank for each tube was used to which was added 1 ml. 2 % (w/v) Na_2CO_3 instead of 1 ml. acetylacetone (1 % w/v) in 2 % (w/v) Na_2CO_3 (Immers & Vasseur, 1950); the reading obtained from this blank, from 2–8 % of the test, was substracted from the test. Provided great care was taken over the neutralization step, and the acetylacetone (redistilled) and *p*-dimethylaminobenzaldehyde solutions were freshly prepared each day, satisfactory reproducibility of the results was obtainable.

Electron microscopy. For the fixation of samples for electron microscopy two fixatives were used. With dormant and germinated spores of both *Bacillus cereus* and *B. polymyxa* the best results were obtained by using 2 % (w/v) $KMnO_4$ for 90 min. at 22° (Mollenhauer, 1959). Outgrowing spores of *B. cereus* were also fixed with 2 % (w/v) $KMnO_4$, but only for 60 min. at 22°. Outgrowing spores of *B. polymyxa* were best fixed with gluteraldehyde + OsO_4 and this fixative was used for these organisms and also for dormant and germinated *B. polymyxa* spores, to allow comparison with $KMnO_4$-fixed samples. The pellet of organisms was resuspended in 5 % (w/v) glutaraldehyde in 0·1 M-phosphate buffer (pH 7·3) for 90 min. at 4°. The organisms were washed once in cold buffer and then resuspended in 1 % (w/v) OsO_4 solution for 120 min. at 4°. The composition of the OsO_4 solution was 5 ml. of 0·14 M-veronal acetate buffer (pH 9·0), 7 ml. of 0·1 N-HCl, 0·12 ml. of 2 M-$CaCl_2$ and 13 ml.

of 2% (w/v) OsO_4. Ten-ml. volumes of fixative solutions were used for 10^8–10^9 organisms. After fixation the organisms were embedded in Epon 812, wet sectioned on a Porter Blum MT 2 microtome, stained with 1% uranyl acetate for 10 min. and examined in a JEM 6S electron microscope.

RESULTS

The amounts of dipicolinic acid, calcium and hexosamine in the dormant spores of *Bacillus cereus* and *B. polymyxa*, are recorded in Table 1, with the percentages lost on germination in the germination media.

Table 1. *Amounts of dipicolinic acid, calcium, and hexosamine in dormant spores of Bacillus cereus and B. polymyxa, and the percentages lost on germination*

		% of spore dry wt.	% lost on germination
B. cereus	Dipicolinic acid	8·4	95–100
	Calcium	4·5	63
	Hexosamine	6·8	32
B. polymyxa	Dipicolinic acid	8·5	95–100
	Calcium	3·5	69
	Hexosamine	7·3	41

Table 2. *Stages of germination and outgrowth of Bacillus cereus spores incubated in nutrient medium*

Time of incubation (min.)	Stage of development	Size of phase-dark forms
0	99% phase-bright, 1% dark	1·0 × 1·0 μ
10	20% phase-bright, 80% dark	2·0 × 1·5 μ
20	14% phase-bright, 86% dark	60% elongated to 3·0 × 1·5 μ
30	15% phase-bright, 85% dark	70% elongated to 3·0 × 1·5 μ
40	15% phase-bright, 85% dark	70% elongated to 4·0 × 1·5 μ
50	10% phase-bright, 90% dark	85% elongated to 5·0 × 1·5 μ
60	12% phase-bright, 88% dark	40% elongated to 6·0 × 1·5 μ, 40% outgrowing from spore coats, and 20% dividing vegetative forms

Table 3. *Stages of germination and outgrowth of Bacillus polymyxa spores incubated in nutrient medium*

Time of incubation (min.)	Stage of development
0	100% phase-bright
35	31% phase-bright, 69% dark
70	31% phase-bright, 45% dark and swollen, 20% showing early outgrowth, 4% vegetative forms
90	33% phase-bright, 19% dark and swollen, 8% showing early outgrowth, 33% outgrown, 8% vegetative forms
120	33% phase-bright, 7% dark and swollen, 6% showing early outgrowth, 5% outgrown, 45% vegetative forms

The stages of germination and outgrowth reached by the organisms after incubation for fixed times in the full nutrient medium are shown in Tables 2 and 3. After 10 min. *Bacillus cereus* spores had germinated; with spores of *B. polymyxa* this took 35 min.

Largely because of the higher concentrations of organisms used with the full nutrient medium, a large proportion of the spores remained dormant throughout the period of incubation; i.e. 10–15 % of *B. cereus* and 30–33 % of *B. polymyxa*. During outgrowth of *B. cereus* spores, the phase-dark organisms first swelled slightly and then elongated to 6 times the length of the dormant spore, before finally growing out from as much of the exosporium and spore coats as remained. On the other hand, *B. polymyxa* spores swelled slightly, and then changed to a crescent shape before early growth was evident from the distortion at the central portion of the outside surface of the crescent. The organisms were considered to have grown out when the virtually complete vegetative bacillus was seen with the spore integument still clearly visible, generally as a figure 3 at one end of the rod. After the first division occurred the organisms were said to be vegetative.

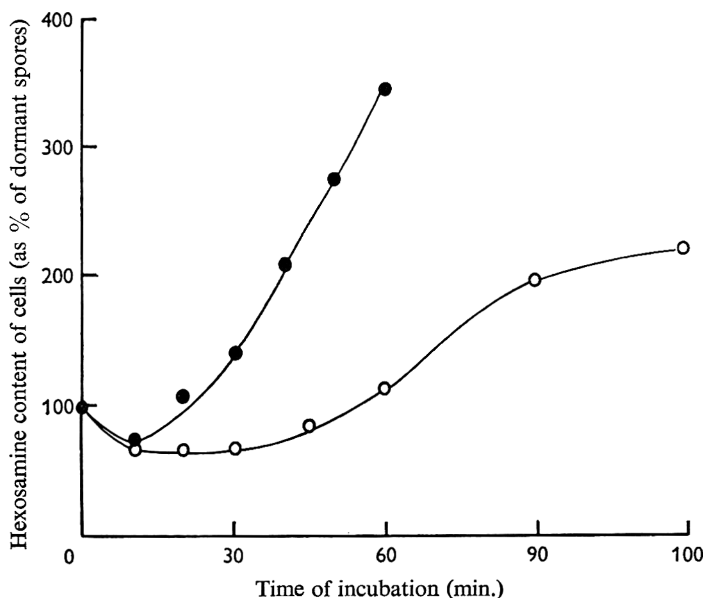

Fig. 1. Hexosamine content of *Bacillus cereus* and *B. polymyxa* spores incubated in nutrient medium. The results are expressed as percentages, the hexosamine content of the dormant spore being taken as 100%. ●, *B. cereus*; ○, *B. polymyxa*.

The results in Fig. 1 show the hexosamine content of organisms taken from incubations in the nutrient medium. Under these conditions, the loss of hexosamine associated with germination was 30 % for *Bacillus cereus* and 33 % for *B. polymyxa*. These values agree favourably with the values of 32 % and 41 %, respectively, for germination in the chemically defined germination media (Table 1). From Fig. 1 we can see that the re-incorporation and resynthesis of mucopeptide associated with the cell-wall synthesis of the vegetative form had begun after 10 min. with *B. cereus* and after 30 min. with *B. polymyxa*, i.e. immediately after germination was completed.

Rode, Lewis & Foster (1962) showed the effectiveness of $KMnO_4$ as a fixative for dormant spores; in our hands also this fixative gave satisfactory results with these organisms. In contradiction with these authors, however, the cortex was clearly visible in our $KMnO_4$ preparations. Although $KMnO_4$ proved the best fixative for *Bacillus*

cereus throughout germination and outgrowth, the most satisfactory results with *B. polmyxa* after germination were obtained using glutaraldehyde + OsO_4.

On Pl. 1 and 2, fig. 1–8, are shown spores of *Bacillus cereus* taken from samples incubated in nutrient medium. Plate 1, fig. 1, shows a spore with the exosporium, three spore coats, cortex, cortical membrane or core wall, core or plasma membrane, and core, all clearly visible. There is evidence of a laminated structure in the exosporium and in the middle spore coat, and a very definite laminated structure in the inner coat. The outer coat appears much more granular in its structure. Between the inner spore coat and the cortex there is a dark diffuse layer. The core is bounded by a plasma membrane of characteristic unit membrane structure, and the core wall is also present. The core itself appears as a uniform dense background with areas containing collections of large granules approximately 100 Å in diameter. There is no evidence of any nuclear area.

Plate 1, fig. 2 and 3, show spores after 2 and 10 min. incubation in the nutrient medium. Germination occurred very rapidly and was associated with the following changes in structure. The cortex has disappeared entirely and the core swollen to fill the whole volume of the cell inside of the spore coats. The organization of the core has altered to show the granular network of ribosomes characteristic of the vegetative form. In Pl. 1, fig. 3 the development has proceeded further and nuclear areas are evident in the cytoplasm; also, the spore coats have drawn away slightly from the core or developing vegetative form. The cytoplasm is bounded solely by the plasma membrane and there is no evidence for the continued existence of the core wall. This separation of the plasma membrane from the spore coats is more evident after 30 min. (Pl. 1, fig. 4). At this stage we can also see the extensive dissolution of the outer and middle spore coats.

Plate 1, fig. 5 and Pl. 2, fig. 6 and 7 show outgrowing forms of *Bacillus cereus* after 40 and 60 min. When the inner spore coat begins to dissolve away, the cell wall and plasma membrane are both present in the outgrowing form. The cortex and middle spore coat are completely absent and, depending on the section, fragments of the inner coat, or inner and outer coats, or both coats plus the exosporium, are seen round one end only of the outgrowing form. Plate 2, fig. 8 shows an example of the integument fragments left behind by the outgrown forms.

On Pl. 3 and 4, fig. 9–16 are a similar series of pictures of germinating and outgrowing spores of *Bacillus polymyxa*. The only differences evident between the *B. polymyxa* spore (Pl. 3, fig. 9) and the *B. cereus* spore (Pl. 1, fig. 1) are in the exosporium and spore coats. *Bacillus polymyxa* lacks an exosporium and has a very different coat structure. The smooth outer surface consists of a bimolecular leaflet type structure, beneath which lies an undifferentiated area and then a highly structured layer of at least 6 bimolecular leaflets or laminates. Between this highly structured layer and the cortex there is the dark structureless layer which is also seen in *B. cereus* spores. The cortex, core wall, plasma membrane and core are the same as those found in *B. cereus*.

Plate 3, fig. 10 shows that in *Bacillus polymyxa* the only changes which occur with germination are in the core, which develops the characteristic granular appearance of the cytoplasm of the vegetative form and a nuclear area. This picture was taken from spores germinated in the chemically defined germination medium, but the same changes were noted in spores germinated by 35 min. incubation in the full nutrient medium.

Plates 3 and 4, fig. 11–16 show preparations (fixed with glutaraldehyde + OsO_4,) of spores germinated in the germination medium, and of spores growing out after 50, 70 and 90 min. in the nutrient medium. Comparing fig. 11 and 12 with fig. 9 and 10 on Pl. 3, we can see that this method of fixation shows less structure in the spore coat and core, but gives a very good picture of the cortex, core wall and plasma membrane. The cortex appears to consist of two layers clearly separated from each other by a dark line. Again the only observable difference after germination is in the core organization. Plate 3, fig. 13 and 14 show organisms at the stage of early outgrowth. The coat and cortex, still apparently largely unaltered, are splitting open at one site in the equatorial region. As this process continues, the core wall develops from a thin dark line to the complete cell wall of the vegetative form in the almost fully outgrown organism in Pl. 4, fig. 15. Plate 4, fig. 16 shows an outgrown form in which the coat and cortex layers appear to have split open round the entire equator. Even with these fully outgrown forms, some cortical material is still present in the spore integument, although a gradual dissolution of the cortex structure can be seen in the development of germinated to outgrown forms.

DISCUSSION

These series of electron micrographs show the different characteristics of the germination and outgrowth of *Bacillus cereus* and *B. polymyxa* spores. The results with *B. cereus* are in good agreement with the findings of Gould (1962) and Chapman & Zworykin (1957), and *B. polymyxa* shows all the established characteristics of small-spored species such as *B. subtilis* (Gould, 1962). In particular, there is excellent agreement between our own results and those of Kawata *et al.* (1963), and Rousseau *et al.* (1966).

The cell wall of the vegetative form of *Bacillus polymyxa* clearly develops directly from the core wall of the spore, although the situation is not so clear cut with *B. cereus* where the core wall seems to disappear along with the cortex during germination. In this connexion it is interesting to note there is no evidence of cell-wall synthesis in thin sections of *B. cereus* until after 40 min. incubation in the nutrient medium, although the hexosamine content of the organisms began to increase again after only 10 min. incubation (Fig. 1). With *B. polymyxa*, the cell wall of the vegetative form is not evident in thin sections until after 50 min., although the hexosamine content of the organisms began to increase after 30 min.

It has been claimed that the dipicolinic acid, calcium and mucopeptide of the spore are located in the cortex (Mayall & Robinow, 1957; Warth *et al.* 1963; Vinter, 1965) and that a prime event in germination is a dissolution of the cortex structure (Mayall & Robinow, 1957; Kawata *et al.* 1963). Our findings on the loss of these components from germinated spores of both *Bacillus cereus* and *B. polymyxa* (Table 1) are in agreement with these hypotheses. However, although the solubilization of the diplicolinic acid, calcium and mucopeptide was associated with the disappearance of the cortex in germinated *B. cereus* (Pl. 1, fig. 2), the same process in *B. polymyxa* caused no apparent alteration in its cortical structure (Pl. 3, figs. 10 and 12). With both spores, however, only 30–40 % of the total mucopeptide was solubilized on germination. The 70–60 % which was retained by the organisms seems therefore to have a function quite separate from that of preserving spore dormancy. From our data, this retained mucopeptide

must be entirely or very largely in the exosporium of *B. cereus*, but in the core wall and cortex of *B. polymyxa*.

Although these data do not allow us to suggest any mechanism whereby the mucopeptide, and dipicolinic acid and calcium, preserve spore dormancy, they do serve to highlight the fact that only a certain proportion of the mucopeptide is directly involved in this dormancy, and to illustrate that, while the changes in chemical structure and biological activity associated with germination are virtually identical in the two species, the morphological changes are quite different.

In studies of germination it has been assumed that the only materials lost from the spore are dipicolinic acid, calcium and mucopeptide. Powell & Strange (1953) showed that *Bacillus megaterium* spores lost 30 % of their dry weight on germination, and in our studies we have noted weight losses from 30 % to 50 %. On the basis of present data (Murrell & Warth, 1965) hexosamine can be taken to represent approximately 50 % of the weight of the spore mucopeptide. From the data in Table 1 we see that the loss of dipicolinic acid, calcium and mucopeptide during germination of *B. cereus* amounted to only 16 % of the dry weight of the spore, and of *B. polymyxa* to 17 %. It would appear therefore that dipicolinic acid, calcium and mucopeptide cannot be the only materials lost from the spore on germination.

The authors are grateful to Miss L. F. Parkin for skilled technical assistance.

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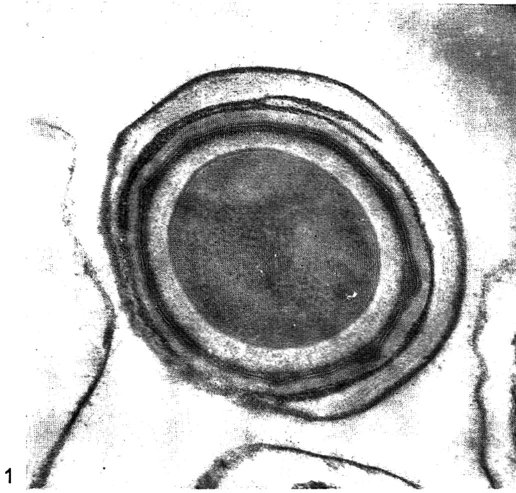

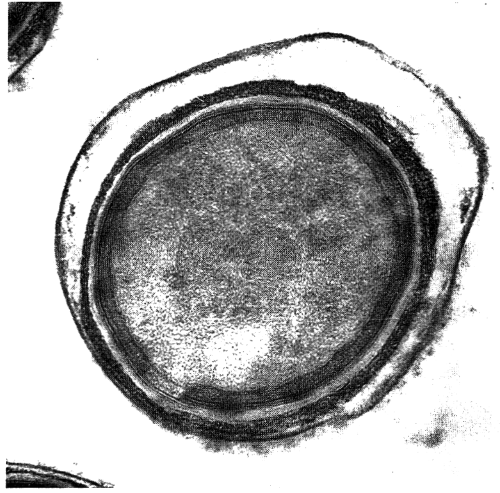

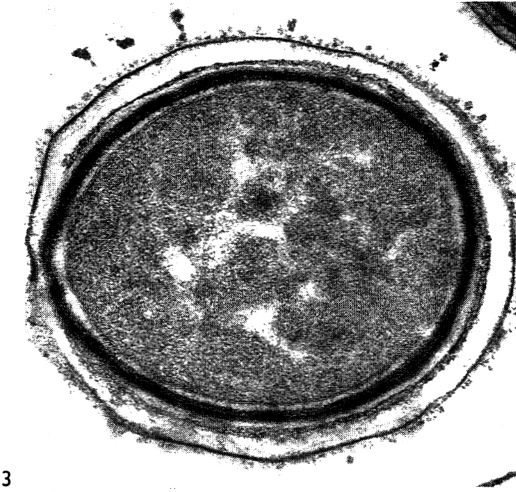

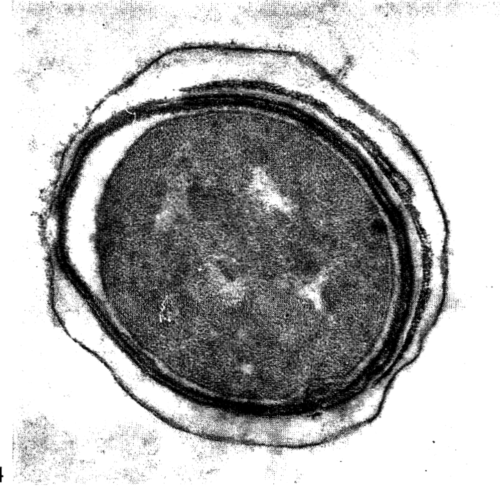

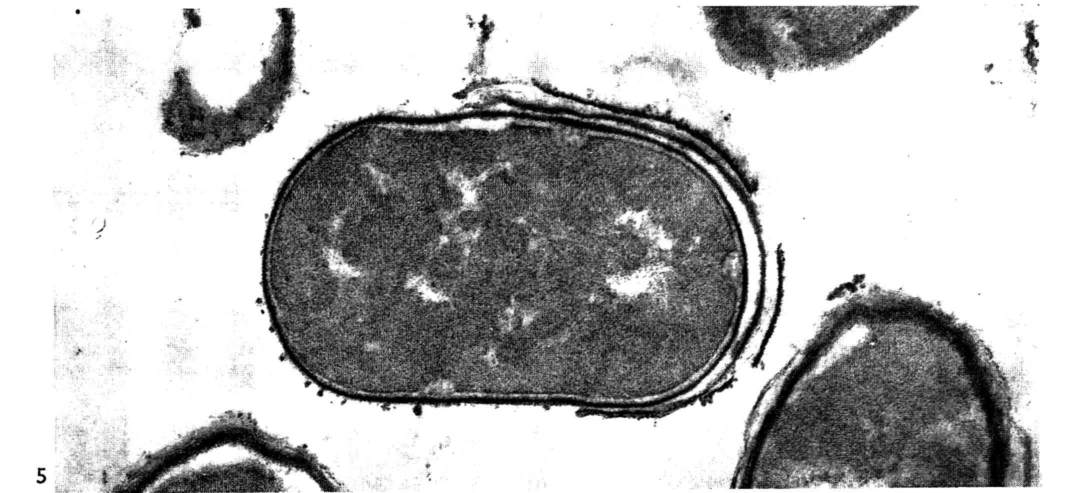

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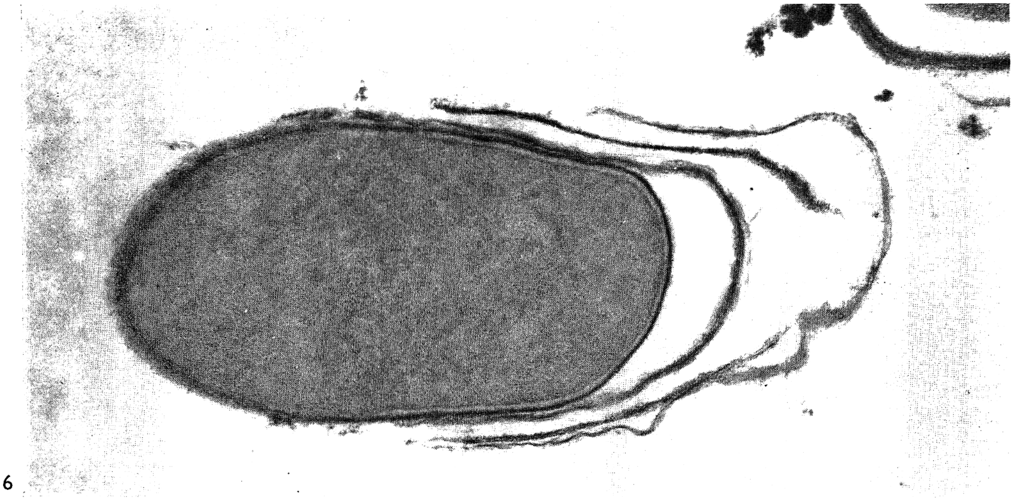
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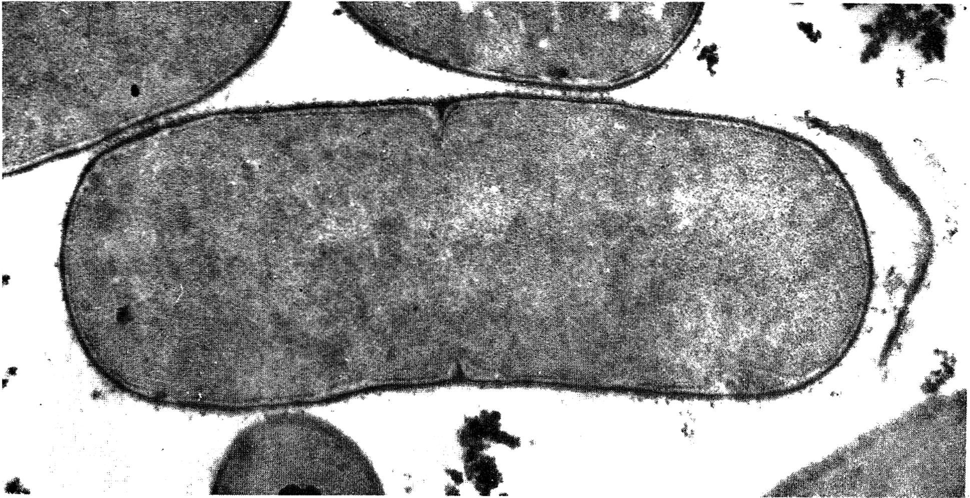
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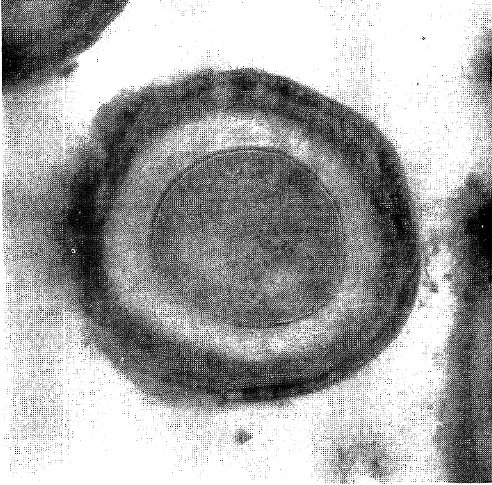
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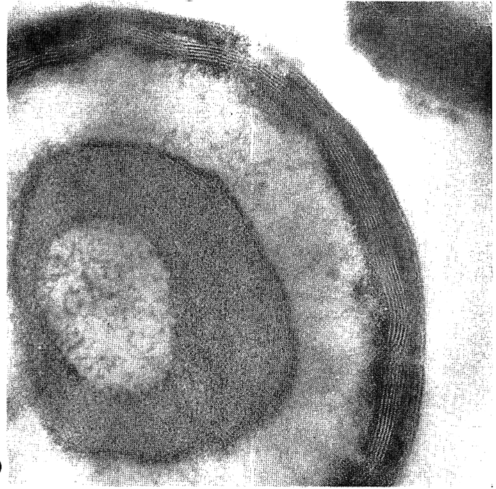
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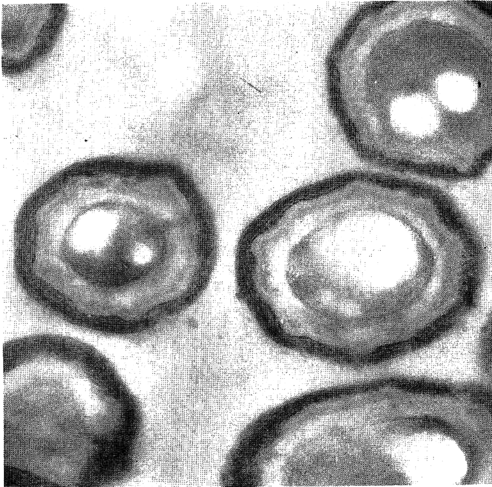
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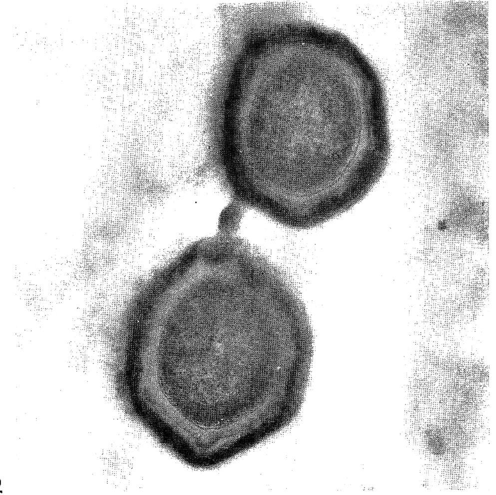
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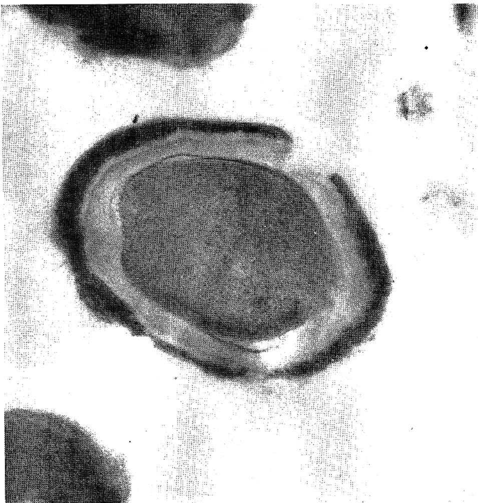
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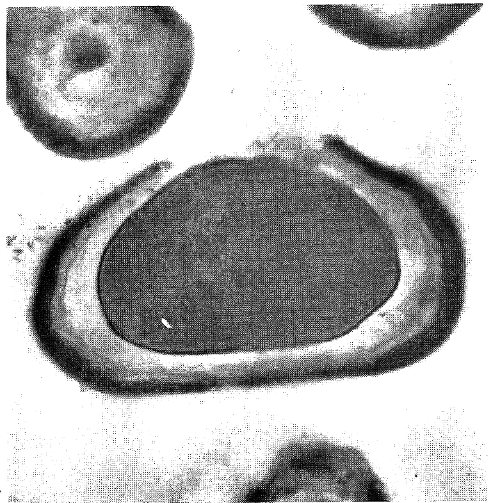
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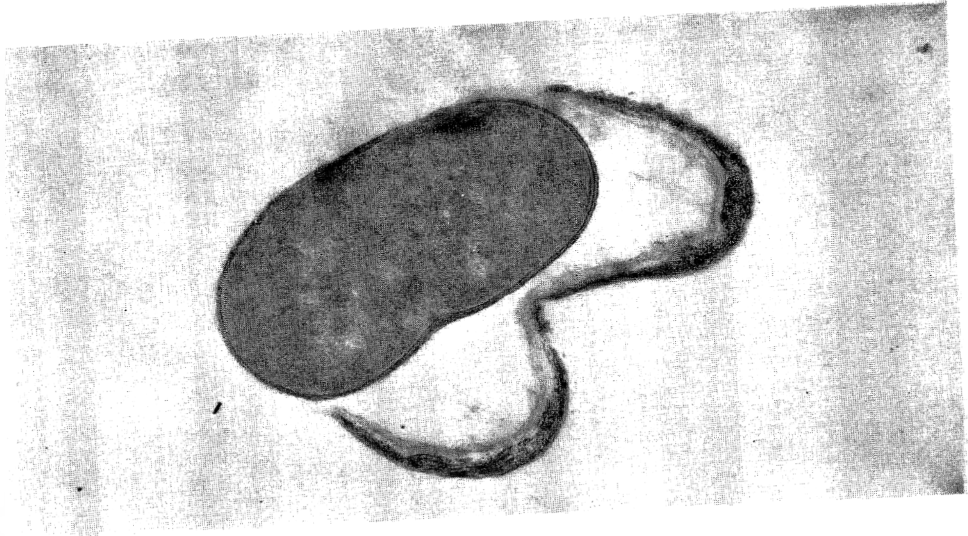
Plate 4



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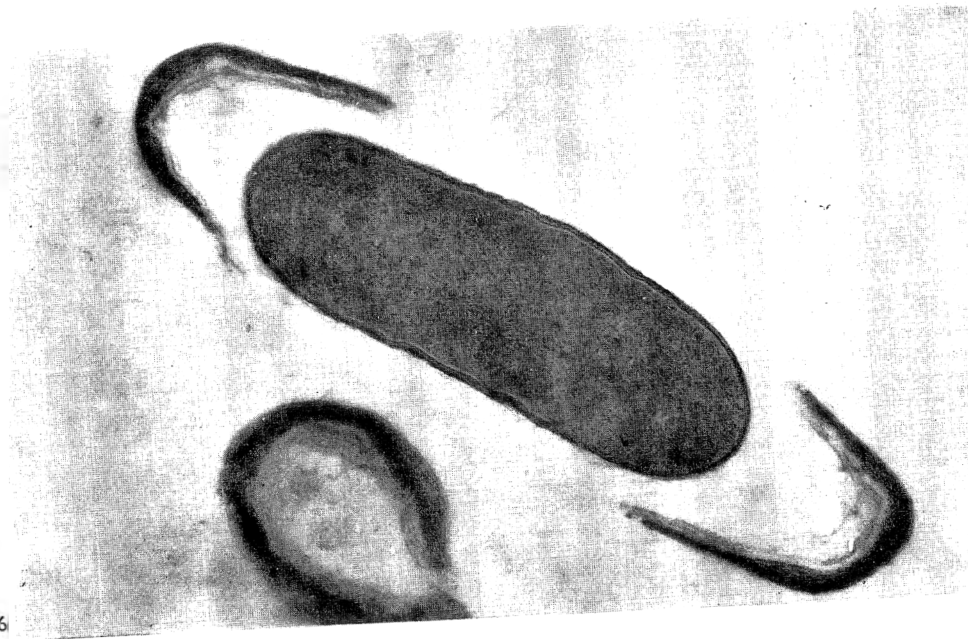

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Thin section of *Bacillus cereus*. Dormant spore; $KMnO_4$ fixed. × 60,000.

Fig. 2. Thin section of *B. cereus*. Germinated spore after 2 min. incubation in nutrient medium; $KMnO_4$ fixed. × 60,000.

Fig. 3. Thin section of *B. cereus*. Germinated spore after 10 min. incubation in nutrient medium; $KMnO_4$ fixed. × 60,000.

Fig. 4. Thin section of *B. cereus*. Germinated spore after 30 min. incubation in nutrient medium; $KMnO_4$ fixed. × 60,000.

Fig. 5. Thin section of *B. cereus*. Outgrowing spore after 40 min. incubation in nutrient medium; $KMnO_4$ fixed. × 40,000.

PLATE 2

Fig. 6. Thin section of *Bacillus cereus*. Outgrowing spore after 40 min. incubation in nutrient medium; $KMnO_4$ fixed. 40,000.

Fig. 7. Thin section of *B. cereus*. Outgrown form after 60 min. incubation in nutrient medium; $KMnO_4$ fixed. × 40,000.

Fig. 8. Thin section of *B. cereus*. Spore integuments left by outgrown forms; $KMnO_4$ fixed. × 20,000.

PLATE 3

Fig. 9. Thin section of *Bacillus polymyxa*. Dormant spore; $KMnO_4$ fixed. × 70,000.

Fig. 10. Thin section of *B. polymyxa*. Spore germinated in chemically defined germination medium; $KMnO_4$ fixed. × 87,500.

Fig. 11. Thin section of *B. polymyxa*. Dormant spore; glutaraldehyde + OsO_4 fixed. × 40,000.

Fig. 12. Thin section of *B. polymyxa*. Spore germinated in chemically defined germination medium; glutaraldehyde + OsO_4 fixed. × 40,000.

Fig. 13. Thin section of *B. polymyxa*. Outgrowing spore after 50 min. incubation in nutrient medium; glutaraldehyde + OsO_4 fixed. × 40,000.

Fig. 14. Thin section of *B. polymyxa*. Outgrowing spore after 70 min. incubation of nutrient medium; glutaraldehyde + OsO_4 fixed. × 40,000.

PLATE 4

Fig. 15. Thin section of *Bacillus polymyxa*. Outgrowing spore after 90 min. incubation in nutrient medium; glutaraldehyde + OsO_4 fixed. × 40,000.

Fig. 16. Thin section of *B. polymyxa*. Outgrowing spore after 90 min incubation in nutrient medium; glutaraldehyde + OsO_4 fixed. × 40,000.

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Production of Thymineless Mutants in Gram-Negative Bacteria (Aerobacter, Proteus)

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SUMMARY

Conditions necessary for the production of thymineless mutants in Gram-negative bacteria by aminopterin treatment were examined. Although *Escherichia coli* and Proteus strains were readily made 'thymineless' (i.e. requiring added thymine) no such mutants were obtained with the majority of Aerobacter strains. However, two methods which produced thymineless mutants in all the Aerobacter strains were found; these were: treatment with alkaline EDTA before, or temperature shift down during, incubation with aminopterin. In every case where temperature shift alone resulted in the production of thymineless mutants more were found when it was used in conjunction with EDTA pretreatment. It was also found that the phase and size of inoculum, the method used to sterilize aminopterin and the concentration of thymine in the plating media had profound effects on the production and detection of thymineless mutants.

INTRODUCTION

Compared with the other nuclear bases thymine is unique in that it is found in DNA but not in RNA; hence thymineless mutants (i.e. requiring added thymine for growth) have proved to be valuable in the study of bacterial DNA. Thymine starvation of such mutants can lead to 'thymineless death' (Barner & Cohen, 1954), the cause of which is not completely known. In other cases thymine deficiency in culture media for thymineless mutants has been shown to induce mutations (Coughlin & Adelberg, 1956) lysogenic bacteriophage (Korn & Weissbach, 1962) colicin production (Sicard, 1961) and premature initiation of the DNA-replication cycle (Pritchard & Lark, 1964). The elimination of 'curing' of extrachromosomal elements from thymineless mutants has been demonstrated under conditions of thymine deprivation (Clowes, Moody & Pritchard, 1965) for sex (F) and various colicin (*col*) factors. Most of these studies have been made on thymineless mutants of *Escherichia coli* which were produced by suitable aminopterin treatment (Okada, Yanagisawa & Ryan, 1961; Clowes *et al.* 1965). During a study of resistance transfer (RT) factors in various species of Gram-negative bacteria it was considered necessary to examine their elimination. Acridine treatment has been shown to eliminate RT factors readily from Shigella and Escherichia (Watanabe & Fukasawa, 1961) but Clowes *et al.* (1965) showed that it did not eliminate certain *col* factors which could be removed by thymine deprivation of thymineless mutants of *E. coli.* These authors concluded that thymine deprivation seems to differentiate factors which cannot be eliminated and are integrated in the chromo-

some, for example, the F factor in Hfr (high frequency of recombination) strains, from those which are extrachromosomal and are eliminated, for example, the F factor in F^+ strains.

METHODS

Organisms. The following strains were used: *Aerobacter cloacae* 53, *A. aerogenes* 418 and 373 and *Escherichia coli* 419 (Smith & Hamilton-Miller, 1963) and *Aerobacter* (*Klebsiella*) *aerogenes* 43, 414 and 415, *Proteus morganii* 185 and *P. rettgeri* 410 (Hamilton-Miller, Smith & Knox, 1965). *Escherichia coli* K 12 met^- was supplied by Professor W. Hayes, *E. coli* 114 was a strain isolated *post mortem*.

Media. Davis Mingioli (DM) medium was used throughout this work (Davis & Mingioli, 1950). The mineral salts solution and the glucose solution were sterilized separately by autoclaving (115° for 10 min.). Solid DM medium was made by mixing 50 ml. cold double strength DM medium with 1·5 g agar in 40 ml. water which had been melted by autoclaving, and other additions or 10 ml. water were added before the plates were poured. The only organism which required a supplement was *Escherichia coli* K 12, and when this strain was examined L-methionine at 20 μg./ml. was used.

Reagents. Thymine and aminopterin were obtained from L. Light and Co. Ltd., Colnbrook, Bucks. Thymine was made up as a 2 mg./ml. solution in distilled water and sterilized by autoclaving. Aminopterin was initially sterilized by autoclaving, but in later experiments it was dissolved in sterile double strength DM medium, then thymine and water added and sterilization effected by filtration through a no. 5 sintered glass filter. Sterility tests were always done.

Method for production of thymineless mutants. Details of the experimental procedures used are given for each experiment under the appropriate tables.

Estimation of reversion rates to thymine independence. Cultures of thymineless mutants of each organism were grown overnight in DM medium containing 80 μg. thymine/ml.; 1·0 ml. was spread on 10 DM agar plates without added thymine and incubated overnight. Serial decimal dilutions made in DM buffer containing 80 μg. thymine/ml. were also counted on DM agar containing 80 μg. thymine/ml. From the counts obtained on DM medium without added thymine the number of revertants/ml. was obtained. This was used to calculate the frequency of reversion per bacterium growing on DM agar containing 80 μg. thymine/ml.

RESULTS

Aerobacter cloacae 53 was incubated for 2 days at 37° in 0·8 mM-aminopterin + 200 μg. thymine/ml. in DM medium and examined for thymineless mutants; none was obtained (limit of detection 0·2 %). Higher concentrations of aminopterin up to 2 mM were tested in four experiments without success. *Escherichia coli* organisms are generally impermeable to charged molecules for which there is no transport system, but it has been shown that EDTA treatment increases their penetration (Leive, 1965). The effect of EDTA on the production of thymineless mutants by growth in aminopterin was therefore examined. An 18 hr culture of *A. cloacae* 53 was diluted 10^{-4} in 0·05M-phosphate buffer (pH 8) containing mM-EDTA and incubated for 15 min. at 37° (EDTA pretreatment). The bacteria were then diluted 1/10 into DM medium containing 0·8 mM-aminopterin + 200 μg. thymine/ml., incubated for 2 days at 37° and

varying dilutions plated on DM agar containing 200 μg. thymine/ml. After overnight incubation those plates which showed discrete colonies were then examined for thymineless mutants by replication to DM agar without added thymine; a low proportion (0·83 %) of these was obtained.

These mutants were taken and investigated to check what concentration of thymine was required for survival. It was found (Table 1) that in liquid media large inocula survived better than small inocula in any one concentration of thymine. Presumably organisms of the large inocula did so because of redistribution of excess intracellular thymine, whereas this thymine was diluted out in small inocula.

Table 1. *Effect of inoculum size on thymineless death of a thymineless mutant of Aerobacter cloacae 53 in various sub-optimal concentrations of thymine*

A thymineless mutant of *Aerobacter cloacae* 53 was grown overnight in DM medium containing 200 μg. thymine/ml. and various dilutions in DM medium containing different concentrations of thymine made. After incubation at 37° for 6 hr each culture was counted on DM agar containing 200 μg. thymine/ml.

Inoculum (bacteria/ml.)	Concentration of thymine (μg./ml.)			
	10	5	2·5	1·25
	Survival (%)			
7×10^6	> 100*	> 100†	34·7	2·8
7×10^4	69·4	8·3	2·8	0·8
7×10^2	37·5	< 2	< 2	< 2

* Multiplied 19-fold. † Multiplied 6-fold.

Table 2. *Effect of thymine concentration in plating media on detection of thymineless mutants of Aerobacter cloacae 53*

Cultures in DM medium of *Aerobacter cloacae* 53 and a thymineless mutant of the same strain were mixed. Viable counts were made before mixing. From these counts the content of thymineless mutants in the mixture was 23·2 %. Dilutions were spread on various concentrations of thymine in DM agar and incubated overnight. Plates showing discrete colonies were taken and replicated on the same concentration of thymine and on to plates without thymine and the number of mutants scored after overnight incubation.

Concentration of thymine (μg./ml.)	Thymineless mutants detected (%)	Proportion of thymineless mutants recovered
30	22·8	0·98
50	24·3	1·05
100	12·5	0·54
150	8·3	0·36
200	6·1	0·26

The need for thymine in solid media was examined; it was found that 30 μg. thymine/ml. was the optimum whereas with 15 to 25 μg. thymine/ml. the colonies were smaller and no growth at all occurred at 10 μg./ml. As the optimum concentration of thymine was 30 μg./ml. it seemed possible that the 200 μg. thymine/ml. used in the solid media in the production of thymineless *Aerobacter cloacae* 53 mutants may have decreased the degree of their detection. To test this, wild-type bacteria were mixed

with thymineless bacteria in a ratio of 3:1 and plated on various concentrations of thymine. After incubation overnight each plate was replicated on to a plate containing the same amount of thymine as well as to a plate without thymine and the number of thymineless mutants scored. The results (Table 2) showed that concentrations of thymine above 50 μg./ml. allowed some mutant clones to grow when replicated on to DM medium without thymine and at 200 μg./ml. only about 25 % of the thymineless mutants were detected. Thus in all subsequent experiments 30 μg. thymine/ml. was used in the solid media to prevent thymineless mutants from escaping detection; also 30 μg. thymine/ml. was included in the diluent to prevent thymineless death.

The phase of growth, the dilution of the inoculum, the method of sterilization of the reagents and the effect of EDTA pretreatment in the production of thymineless mutants of *Aerobacter cloacae* 53 were then examined. No mutants were detected in any experiment where EDTA pretreatment was omitted. The results of the EDTA pretreatment series (Table 3) showed that autoclaving (115° for 10 min.) decreased the activity of aminopterin as compared with filtration. The results also showed that when bacteria in the logarithmic phase were used as inoculum more mutants were obtained than when stationary phase bacteria were used. Furthermore, the inoculum size was important: the smaller the inoculum the greater the proportion of thymineless mutants obtained. Since all cultures reached about 2×10^8 bacteria/ml. it seems that many generations in the presence of aminopterin were necessary before an organism mutated to thymineless.

Table 3. *Effect of age, size of inoculum, and method of sterilization of aminopterin on the production of thymineless mutants of Aerobacter cloacae 53*

A 3 hr and an 18 hr culture of *Aerobacter cloacae* 53 in DM medium were suitably diluted into 0·05 M-phosphate buffer (pH 8·0) with and without mM-EDTA and incubated at 37° for 15 min. then diluted into DM medium containing 0·8 mM-aminopterin and 200 μg. thymine/ml. The experiment was done in duplicate with either filter-sterilized or autoclaved aminopterin solution. Incubation in aminopterin + thymine in DM medium was made for 2 days at 37° before plating on to DM agar containing 30 μg. thymine/ml. and screening for thymineless mutants by replication on to DM agar with and without 30 μg. thymine/ml. The results with EDTA pretreated bacteria only are shown since no mutant was detected where pretreatment was done in phosphate buffer only. Limit of detection, 0·5 %.

Phase of organisms inoculated	Method used to sterilize aminopterin solution	Approximate inoculum (viable bacteria/ml.) 10^3 — Thymineless mutants (%)	Approximate inoculum (viable bacteria/ml.) 10^5 — Thymineless mutants (%)
Stationary (18 hr culture)	Autoclaving	4·3	0
	filtration	22·1	0
Logarithmic (3 hr culture)	Autoclaving	12·3	0
	filtration	80·2	0·7

The effect of incubation temperature without the use of EDTA pretreatment was examined by using small inocula of logarithmic-phase *Aerobacter cloacae* 53 organisms and filter-sterilized aminopterin solutions. Ten incubation temperatures between 23° and 41° were tested but no thymineless mutants were found. However, during these experiments a sample of culture incubated at 37° on the first day was re-incubated at 35° on the second day by mistake and in this case 76·7 % of the bacteria were found to be thymineless mutants. Therefore the effect of temperature shift was investigated.

The results (Table 4) showed that decreasing the incubation temperature from 37° to 35° after the first day produced a large proportion of thymineless mutants, whereas any larger decrease of incubation temperature decreased the number of mutants and any increase in incubation temperature gave no mutants at all. The growth rates of *Aerobacter cloacae* 53 and a thymineless mutant of the same strain in DM medium containing 200 μg. thymine/ml. were investigated at 37° and 35°. The generation time of the parent strain was increased from 78 min. at 37° to 83 min. at 35°, whereas the generation time of the thymineless mutant was decreased from 84 min. at 37° to 79 min. at 35°. Thus the multiplication of the thymineless mutant was somewhat temperature-sensitive as compared with that of the wild type. Incubation with aminopterin for 2 days at 37° or 35° did not give any thymineless mutants, whereas incubation at 37° for the first day and 35° for the second day did produce thymineless mutants. Therefore it seems that an incubation temperature of 37° is essential to promote the formation of thymineless mutants which, once formed multiply more rapidly at 35° and hence can be detected in significant amounts.

Table 4. *Effect of incubation temperature on the production of thymineless mutants of Aerobacter cloacae 53*

A 3 hr culture of *Aerobacter cloacae* 53 in DM medium was diluted 10^{-4} into 0·05 M-phosphate buffer (pH 8·0), incubated for 15 min. at 37°, then diluted 1/10 into DM medium containing 0·8 mM-aminopterin – 200 μg. thymine/ml. This was incubated for 2 days at various temperatures, diluted in DM medium containing 30 μg. thymine/ml. and plated on DM agar containing 30 μg. thymine/ml. and incubated overnight. Plates containing discrete colonies were replicated on to 30 μg. thymine/ml. and 0 μg. thymine/ml. DM agar, incubated overnight and the number of thymineless mutants scored. Limit of detection, 0·5 %.

Incubation temperature (° C.)		Thymineless mutants observed
First day	Second day	(%)
37	35	81·2
37	33	71·0
37	31	0
35	37	0
35	37	0
33	37	0
31	37	0

Thus two systems were found which produced thymineless mutants of *Aerobacter cloacae* 53, both these methods were tested separately and in combination in the same experiment. The results (Table 5, top line) showed that either method increased the proportion of thymineless mutants to about the same extent and that both methods used together gave a still larger proportion of thymineless mutants (94 %). The method was therefore applied to other species of Gram-negative bacteria with the results shown in Table 5. These methods (EDTA pretreatment and temperature shift down) decreased the proportion of thymineless mutants for *Escherichia coli* and Proteus species, but all the Aerobacter strains tested were made thymineless more readily. Indeed with 4 out of 6 of the Aerobacter strains examined no mutants were detected by straightforward aminopterin treatment even though concentrations up to 2 mM were tested. However, the use of EDTA pretreatment and/or temperature shift induced thymineless mutants in all these previously refractory Aerobacter strains, although

with two of these strains temperature shift did not produce thymineless mutants. EDTA pretreatment was the most consistent method in the production of thymineless mutants in the Aerobacter strains, and in every case where temperature shift down alone resulted in thymineless mutants more were found when temperature shift down was used after EDTA pretreatment.

Table 5. *Effect of temperature shift and EDTA pretreatment on the production of thymineless mutants in various species of Gram-negative bacteria*

Three-hour cultures in DM medium were pretreated in 0·05 M-phosphate buffer (pH 8·0) or in mM-EDTA in phosphate buffer and diluted 1/10 at an inoculum size of about 10^3 bacteria/ml. into DM medium containing 200 μg. thymine/ml. and various concentrations of aminopterin. After incubation for 2 days at the temperatures indicated the cultures were screened for thymineless mutants by plating on DM agar + thymine (30 μg./ml. for all strains except Proteus strains where 80 μg./ml. was used) and replica plating on DM agar with and without thymine. Limit of detection, 0·5 %.

Pretreatment	Pretreatment: Phosphate buffer only		Pretreatment: mM-EDTA in phosphate buffer			
Incubation temperature Day 1 ...	37°	37°	37°	37°		
Day 2 ...	37°	35°	37°	35°		
	Thymineless mutants obtained (%)				Concentration of aminopterin used (mM)	Reversion rate per bacterium per division
Aerobacter cloacae 53	0	87	87	94	0·8	$< 1{\cdot}5 \times 10^{-9}$
A. aerogenes 43	0	0	0	0	0·8	
	0	0	6	0	1·6	$9{\cdot}1 \times 10^{-6}$
	0	0	3	0	2·0	
A. aerogenes 373	0	0	4	0	0·8	$2{\cdot}0 \times 10^{-3}$
	0	0	0	0	1·6	
A. aerogenes 414	18	36	15	98	0·8	$1{\cdot}4 \times 10^{-5}$
A. aerogenes 415	10	19	32	74	0·8	$9{\cdot}1 \times 10^{-6}$
A. aerogenes 418	0	6	0	92	0·8	$< 9{\cdot}1 \times 10^{-10}$
Escherichia coli 114	97	28	0	0	0·8	$< 1{\cdot}4 \times 10^{-9}$
E. coli 419	6	4	8	0	0·8	$4{\cdot}2 \times 10^{-8}$
E. coli K12	99	96	2	53	0·8	$< 4{\cdot}0 \times 10^{-9}$
Proteus morganii 185	100	33	0	0	0·8	$1{\cdot}2 \times 10^{-7}$
P. rettgeri 410	100	0	0	0	0·8	$1{\cdot}1 \times 10^{-6}$

CONCLUSIONS

The data presented above indicate that optimum conditions for the selection of thymineless mutants in Aerobacter species include: aminopterin should be sterilized by filtration; organisms in the logarithmic phase should be used in small inocula of about 10^3/ml.; thymine near the optimal concentration for growth of thymineless mutants should be used in the plating medium since higher concentrations can decrease the sensitivity of their detection. With the Aerobacter and *Escherichia coli* strains studied this concentration was about 30 μg. thymine/ml.; however, the Proteus species required 75 μg./ml. Nevertheless, with these improved conditions the Aero-

bacter strains were more refractory to aminopterin treatment than were the *E. coli* or Proteus strains as regards the production of thymineless mutants. However EDTA pretreatment before and temperature shift down during aminopterin treatment induced the production of thymineless mutants in all the Aerobacter strains. It is interesting to note that with *A. aerogenes* 43 and 373 increase in aminopterin concentration alone did not result in the production of thymineless mutants whereas EDTA pretreatment at a lower concentration did produce mutants. Thus the effect of EDTA was not a simple one since it did not seem to work solely by increasing the effective intracellular concentration of aminopterin. The resistance of mammalian cells to amethopterin is not due to lack of permeability but seems to be due to higher intracellular concentrations of folic acid reductase which firmly binds the drug (Hakala, 1965). Perhaps, since EDTA increases the permeability of Gram-negative bacteria (Leive, 1965), it may potentiate the effect of aminopterin by causing the bacteria to lose factors like folic acid reductase which would normally block the mutagenic action of aminopterin.

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Effect of Clover Phyllody Virus on Nodulation of White Clover (*Trifolium repens*) by *Rhizobium trifolii*

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SUMMARY

When inoculated with a clone of Rhizobium that produced large pink effective root nodules in virus-free plants, white clover plants infected with clover phyllody virus (CPV) produced mainly small white nodules characteristic of reduced effectiveness in nitrogen fixation. Bacteria from nodules borne on these CPV-infected plants again produced mainly small white nodules in virus-free clover cuttings and seedlings, but did not transmit CPV. Either exposure to the virus, or culture in CPV-infected tissues, seemed to induce a change in the Rhizobium.

INTRODUCTION

Clover phyllody virus (CPV = strawberry green-petal virus) is transmitted in the persistent manner by jassid vectors and causes yellows-type diseases in many legumes and other plants, including a range of annual weeds (Frazier & Posnette, 1957; Chiykowski, 1962; Posnette & Ellenberger, 1963). The disease is particularly prevalent in white clover and in this species, as in other clovers, the inflorescences become transformed into leafy structures which set no seed. Other symptoms include: chlorosis, vein clearing and bronzing of the foliage, diminished leaf size, a degree of stunting and axillary proliferation. Expression of floral symptoms is associated with hormonal unbalance induced by the virus (Carr, 1961) and is linked with zinc metabolism (Carr & Stoddart, 1963).

Surveys (Carr & Large, 1963) and work with experimental plots (Carr & Catherall, 1964) have established the extent of infection and loss of seed and herbage due to CPV. While much of this loss is undoubtedly attributable to the direct effect of virus on host, many of our unpublished observations suggest an indirect effect also—through the interaction of CPV with the clover/Rhizobium symbiosis leading to a degree of ineffective nodulation. The ultimate criterion of ineffectiveness is total or partial failure of nitrogen fixation, but certain associated phenomena are known (Fred, Baldwin & McCoy, 1932). These include: absence of pink pigmentation and the production of many small round nodules over the entire root area rather than of a few large obovate ones restricted mainly to the upper part of the root system. Vanderveken (1964) noted that in CPV-infected white clover, the reaction between an effective bacterial strain and the host was similar to that produced by an ineffective strain on healthy plants, and reported that, when bacteria were re-isolated from infected and transferred to healthy plants, full effectiveness was restored.

The present paper reports on the relationship between CPV and nodulation by a normally highly effective isolate of Rhizobium from white clover, with specific reference to residual effects on transference of bacteria from infected to healthy plants.

METHODS

Sources of materials. The four CPV isolates (here designated B, C, E, F) were chosen from among several obtained originally from a single field-infected white clover plant. Transfer by the natural insect vector, *Euscelis plebejus*, to a range of white clover genotypes randomly selected from the variety S. 100 has established that these isolates vary in the severity and in the type of vegetative symptoms produced, and that there is a large host genotype/virus isolate interaction (Joshi & Carr, unpublished). Throughout this paper the term 'infected' refers to virus infection exclusively and not to infection with Rhizobium. The culture of *Rhizobium trifolii* was derived by dilution plating and re-isolation of a single colony from the highly effective w4 strain in the Welsh Plant Breeding Station collection. This procedure should have given reasonable assurance of a clonally pure line free from accumulated mutants, although there is a chance that the colony may have arisen from more than one cell. In the latter event, the procedure would still have served to limit the initial genetic variation. Cultures of the Rhizobium w4 were maintained on yeast mannitol agar.

Nodulation techniques. All nodulation studies were made in a nitrogen-free agar culture (Jensen, 1942) according to the general method of Nutman (1954). CPV is not seed-borne and seedlings could therefore not be used in experiments involving infected plants. Instead, vegetatively propagated cuttings of virus-free and CPV-infected plants were used. However, the seedling technique was used for determining residual effects due to the previous Rhizobium source. Cuttings proved to have an advantage over seedlings in limiting host genetic variation, but root growth was more erratic and commonly adventitious roots developed from the end of the stolon in addition to normal growth from the root initial.

Two host genotypes, Nos. 33 and 43, selected on the basis of major differences in their reaction to CPV, were clonally propagated in pots of soil in an insect-free glasshouse (*a*) in healthy condition and (*b*) infected with one of the four virus isolates. Cuttings were obtained by detaching from each plant young, actively growing stolons, bearing a single apical growing point and incipient root not yet broken through the outer tissues. After thorough washing, the cut end of each was sealed with paraffin wax and the whole surface-sterilized in 80% ethanol in water for 1 min. After a rinse in sterile distilled water, they were further sterilized by immersion in 0·1% (w/v) mercuric chloride solution for 3 min. and washed for 5 min. in each of 6 changes of sterile distilled water. They were then planted singly and aseptically on 15 ml. slopes of sterile Jensen agar contained in $20 \times 2{\cdot}5$ cm. tubes to which 10 ml. of sterile liquid Jensen medium of quarter strength had been added. Tubes were maintained in a glasshouse with the lower portions shaded, and when, after about 15 days, viable cuttings had begun to develop roots each was inoculated and returned to the glasshouse.

In the first experiment, in which the nodulating ability of infected and healthy cuttings of the two genotypes was compared, all were inoculated in the root area with a single loopful of an aqueous suspension of an actively growing Rhizobium w4 culture. Ten tubes represented each of the 5 'isolates' (4 infected and 1 uninfected) for each of the 2 genotypes, and the whole was arranged as a randomized block. In the second experiment involving cuttings, in which the effect of Rhizobium source as well as plant condition (healthy or infected) on nodulation patterns was under

investigation, transfer was made from a suspension of the bacteria obtained by crushing directly into sterile distilled water three previously surface-sterilized nodules of appropriate origin. Uninfected cuttings, and those infected with CPV-isolate B only, were inoculated with Rhizobium derived both from uninfected and from CPV-isolate B-infected cuttings from the first experiment; each cutting was inoculated with Rhizobium from its own genotype only. Three tubes represented each individual treatment and all treatments were replicated three times in a randomized block.

For the seedling experiment, seeds of the variety S. 100, surface-sterilized previously with 0·1 % (w/v) mercuric chloride solution, were planted singly and aseptically on 7 ml. slopes of sterile Jensen agar contained in 15 × 1·5 cm. tubes, to which 2 ml. of quarter strength sterile Jensen liquid medium had been added. After about 7 days, the young seedlings were inoculated in the root region with a loopful of an aqueous suspension of the appropriate Rhizobium culture. The various Rhizobium cultures were derived from single colonies arising from plating out the contents of nodules borne on cuttings from the first experiment; owing to the failure of an earlier attempt at inoculation, these cultures had been maintained on yeast mannitol agar for more than 4 months, with four successive transfers to fresh medium. Cuttings of genotype 43 only, both in uninfected condition and infected with all four CPV isolates, provided the sources of Rhizobium. Since it seemed possible that the size of nodule from which the rhizobia were isolated might influence subsequent nodulation behaviour and so confuse the main effects, separate isolations of rhizobia were made from large, medium and small nodules for each treatment. Direct inoculation from an original Rhizobium w 4 culture of comparable age served as an overall control, but was excluded from the final analysis as it proved not to differ significantly from the treatment of greatest mean value. Two tubes represented each treatment and these were replicated 3 times in a randomized block in the glasshouse.

Collation of data. Observations of the time of nodule development were made initially at daily intervals and subsequently every 2 weeks up to harvest time 6–8 weeks after inoculation. At these times, nodules were classified into large, medium and small on a purely visual estimate and the number in each class recorded. The statistical technique used in the analysis was such that the variances of individual components (time of sampling, genotype, plant condition, source of Rhizobium) were a measure of variation in the total number of nodules produced, whereas the interaction of any one of these components with size was a function of the frequency of distribution of the nodules over the three classes, large, medium and small. Note was taken of host-plant appearance, of the distribution, shape and colour (white or pink) of the nodules and of the time taken to develop pigmentation. Finally, all plants were removed from the tubes, air dried at 80° for 6 hr and the dry weight recorded. Initially, a chemical determination of total nitrogen was also made, but since dry weight and nitrogen content were found to be correlated completely ($r = +0{\cdot}97$) this practice was subsequently discontinued.

Possible transfer of virus. In the second cutting experiment four additional tubes were included for each healthy genotype inoculated with rhizobia from an infected source, and two additional tubes inoculated with rhizobia from a healthy source served as controls. These additional tubes did not feature in the main experiment, but at harvest time the plants were transplanted from them into pots of steam-sterilized soil and maintained in an insect-free glasshouse until the onset of flowering, when

each was inspected for symptoms of CPV. A representative number of plants from the seedling experiment received similar treatment.

RESULTS

Effect of CPV on growth and nodulation

The data for dry weight (Table 1) show that healthy plants reared from cuttings significantly outyielded infected ones ($P = 0{\cdot}001$), but differences between individual virus isolates and the host genotypes were not significant. Observations of leaf size and mortality in healthy and infected plants indicated that the latter produced a greater number of much smaller leaves ($P = 0{\cdot}05$) which, however, became senescent more rapidly ($P = 0{\cdot}001$). Genotypes differed significantly in this respect, no. 43 producing more leaves of greater longevity than no. 33 ($P = 0{\cdot}01$).

Table 1. *Mean dry weight (in mg.) of healthy and CPV-infected cuttings of 2 white clover genotypes, grown in Jensen agar culture and inoculated with effective w4 culture of Rhizobium trifolii*

Host Genotype no.	Uninfected	Infected with CPV isolate: B	C	E	F	Mean	L.S.D. at 5%
33	52·1	30·4	38·6	36·7	34·8	38·5	N.S.
43	54·4	36·9	39·8	30·8	29·4	38·3	
Mean	53·2	33·6	39·2	33·7	32·1	—	
L.S.D. at 5%		8·9 (B–F)					12·5

Table 2. *Mean time in days to appearance and subsequent colour-change of the first-formed nodule produced on healthy and CPV-infected cuttings of 2 white clover genotypes, grown in Jensen agar culture and inoculated with effective w4 culture of Rhizobium trifolii*

Nodule type	Genotype	Uninfected	Infected with CPV isolate: B	C	E	F	Mean	L.S.D. at 5%
White	33	9·1	14·8	17·8	16·2	18·5	15·3	1·6
	43	12·2	19·4	18·7	18·9	23·5	18·5	
Mean		10·6	17·1	18·2	17·5	21·0	—	—
L.S.D. at 5%			2·6 (B–F)					3·7
Pink	33	13·0	17·7	18·9	18·0	20·5	17·6	1·7
	43	15·9	21·3	19·9	20·9	25·9	20·8	
Mean		14·4	19·5	19·4	19·4	23·2	—	—
L.S.D. at 5%			2·7 (B–F)					3·8
White to pink (by difference)	33	3·9	2·9	1·1	1·8	2·0	2·3	N.S.
	43	3·7	1·9	1·2	2·0	2·4	2·2	
Mean		3·8	2·4	1·1	1·9	2·2	—	—
L.S.D. at 5%			0·7 (B–F)					1·0

There was a significant delay ($P = 0{\cdot}01$) in the time taken for infected cuttings to produce nodules, as compared with that taken by healthy cuttings (Table 2). This was true both for the initial stages and for the overall time taken from inoculation to

the development of pigmentation. However, the time taken to progress from white to pink was substantially shorter for infected than for healthy plants ($P = 0{\cdot}01$). In this essentially continuous process, proportionately fewer nodules produced by infected plants developed pigmentation. Isolate F had a significantly greater effect in delaying nodulation than the other isolates. Genotype 43 took a significantly longer time to nodulate than 33, but there was no genotype/plant condition (healthy versus infected) interaction; differences between genotypes were of the same order in infected and in uninfected plants.

Table 3 presents an analysis of the main components of variation influencing the total number of nodules formed on the plants and their distribution into 3 sizes. To avoid additional complexity of computation, replicates were pooled and the basic error was taken into the third-order interaction. Neither time of sampling nor genotype had any significant influence on nodulation, but plant condition (healthy versus infected) clearly had a major effect both on total number and on distribution. Smaller differences between individual virus isolates were, in this instance, not significant.

Table 3. *Analysis of total number and size distribution of nodules produced by healthy and CPV-infected cuttings of 2 white clover genotypes, grown in Jensen agar culture and inoculated with effective w4 culture of Rhizobium trifolii*

Source of variation	*N*	Mean square
Time of sampling	2	35,846
Genotype	1	6,934
Time × genotype (Error A)	2	3,418
Size	2	55,759 *
Size × genotype	2	7,143
Size × time	4	7,596
Size × time × genotype (Error B)	4	3,547
Isolate		
Healthy *v.* infected	1	3,901 *
Between infected	3	924
Isolate × size		
Healthy *v.* infected	2	18,786 ***
Between infected	6	526
Isolate × size × time		
Healthy *v.* infected	4	2,933 *
Between infected	12	1,204
Residual error†	44	854

† Residual error is compounded of the third order interaction, together with the remaining non-significant first- and second-order interactions.

The nature of the difference in nodule size distribution is illustrated for genotype 33 in the histograms comprising Fig. 1. Fewer total nodules were produced by healthy than by infected plants, but whereas the former had a preponderance of large effective nodules, the latter had a great many small ones which were probably largely ineffective. A high degree of correlation was shown between the number of large nodules produced and dry weight ($r = +0{\cdot}78$), and in subsequent experiments the value for r was even higher. There was some indication that isolates, notably B and E, differed in their effect on nodulation and, on an individual comparison basis, these differences were often significant. However, the general distribution was similar throughout the sampling period.

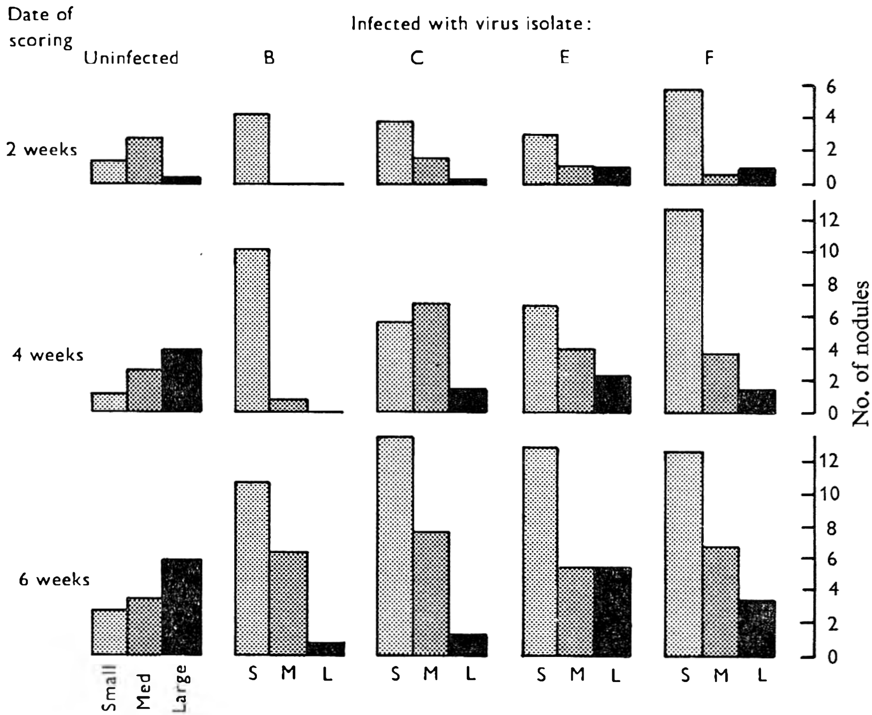


Fig. 1. Size distribution of nodules produced by healthy and CPV-infected cuttings of white clover genotype 33, grown in Jensen's agar culture and inoculated with effective (w4) culture of *Rhizobium trifolii.*

Persistence of ineffectiveness following bacterial transfer. Seedlings. Table 4 shows that both the size of the original nodule and the source plant (healthy or infected) from which the Rhizobium cultures were obtained, influenced significantly the subsequent dry weight of healthy white clover seedlings inoculated with them ($P = 0{\cdot}001$ and 0·01, respectively). Differences between individual isolates were not significant. There was a highly significant interaction between nodule source in respect of size and the source-plant condition, which reference to Table 4 shows to have been due entirely to CPV-isolate E. For the remaining sources (uninfected and infected with

Table 4. *Mean dry weight (in mg.) of healthy S. 100 white clover seedlings grown in Jensen agar culture and inoculated with w4 cultures of Rhizobium trifolii derived from large, medium and small nodules developed on healthy and on CPV-infected plants of genotype 43*

Nodule source (size)	Uninfected	Source of Rhizobium culture: plants infected with CPV isolate: B	C	E	F	Mean	L.S.D. at 5%
Large	14·2	10·1	10·2	9·9	12·7	11·4	1·4
Medium	11·2	9·8	8·7	3·6	4·6	7·6	
Small	3·6	3·9	2·9	11·1	3·4	5·0	
Mean	9·7	7·9	7·3	8·2	6·9	—	—
L.S.D. at 5%		1·8					3·1

CPV-isolates B, C, F) seedling dry weight was conditioned independently both by source-plant condition and the size of the original nodules from which the Rhizobium cultures were derived. Of these factors, original nodule size clearly exercised the major influence, indicating the importance of taking it into account in estimating the effects of other variables. However, the mean values taken over all nodule sizes show that seedlings inoculated with rhizobia from an infected source produced a significantly lower yield than those inoculated with rhizobia isolated from healthy plants: this despite the fact that the cultures had been maintained on agar through four successive transfers before use.

Similar effects were evident in the size distribution of nodules produced by these seedlings (Table 5; Fig. 2). For the sake of simplicity in computation, replicates were again pooled in the detailed analysis but a separate analysis of the main components, which included replicates, showed that error variation was of a low order and that sampling date (time) and the size distribution of nodules were both highly significant.

Table 5. *Analysis of total number and size distribution of nodules produced by healthy S. 100 white clover seedlings grown in Jensen agar culture and inoculated with w4 cultures of Rhizobium trifolii derived from large, medium and small nodules developed on healthy and on CPV-infected plants of genotype 43*

Source of variation	*N*	Mean square
Time of sampling	3	539·6
Size	2	538·6
Time × size (error A)	6	135·8
Treatment		
Nodule source (size)	2	523·4 ***
Isolate		
Healthy *v.* infected	1	2·7
Between infected	3	133·1 ***
Isolate × nodule source		
Healthy *v.* infected	2	112·7 **
Between infected	6	176·4 ***
Treatment × size		
Nodule source × size	4	41·1
Isolate × size		
Healthy *v.* infected	2	387·8 ***
Between infected	6	19·8
Isolate × nodule source × size	16	32·0
Treatment × time	42	10·8
Time × treatment × size (residual error)	84	21·2

Since the relative differences between treatments remained the same whatever the date of sampling (see treatment × time-interaction), only the final (8 week) data are illustrated in the histograms comprising Fig. 2. As with dry weight, so also the total number and size distribution of nodules produced by the seedlings was influenced by the size of nodule from which the rhizobial cultures had been isolated originally but there was no interaction of nodule source with distribution. There was a large degree of nodule source/isolate interaction which reference to the histograms shows to have been due, again, to CPV-isolate E, and in particular to the effect of bacteria isolated from small nodules formed on plants inoculated with this isolate. The anomalous behaviour of CPV-isolate E also accounts for the lack of significance

between the total number of nodules produced on seedlings inoculated with rhizobia from healthy and infected sources. This single observation of virus isolate interaction with rhizobium source should, clearly, be treated with caution. In considering the overall effect of CPV isolate on the distribution of nodule size, differences between healthy and infected sources were highly significant. Although the high between-isolate variation is also due, in part, to the effect of rhizobia isolated from plants infected with CPV isolate E, many individual comparisons between other CPV isolates showed a high order of significance. Figure 1 and Pl. 1, fig. 1, show that these differences were due to the fact that seedlings inoculated with rhizobia from infected plants

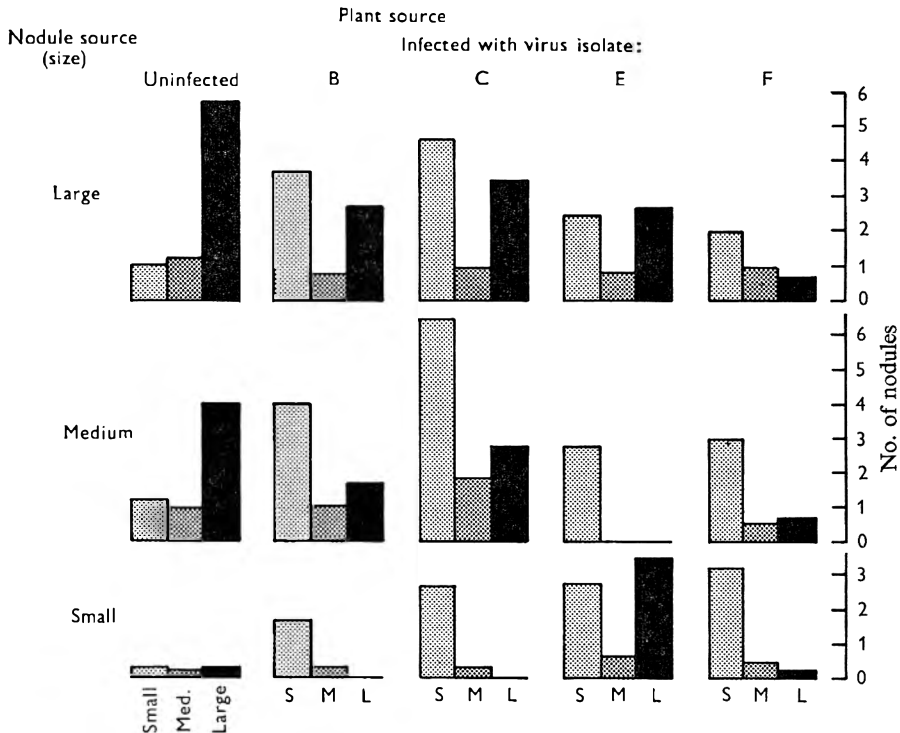


Fig. 2. Size distribution of nodules produced by healthy S. 100 white clover seedlings, grown in Jensen agar culture and inoculated with effective (w4) cultures of *Rhizobium trifolii* derived from large, medium and small nodules developed on healthy and on CPV-infected plants of genotype 43.

produced a larger total number of nodules, but the number of large effective nodules was smaller. Within the virus-infected group, somewhat lesser differences in distribution are also evident, notably between CPV isolates C and F.

Cuttings. In the previously described seedling experiments the nature of the plant material allowed only the effect of Rhizobium source (whether from infected or from healthy plants) on nodulation to be investigated. The use of cuttings has enabled a direct comparison to be made in the same experiment between effects attributable to Rhizobium source and those attributable to plant condition. Table 6 (*a*) shows that the dry weight of these cuttings was affected significantly both by the genotype ($P = 0{\cdot}05$) and by treatment.

Table 6. *Mean dry weight (in mg.) of healthy (H) and CPV-isolate B-infected (P) cuttings of 2 white clover genotypes, grown in Jensen agar culture and inoculated with effective w4 cultures of Rhizobium trifolii obtained from healthy (h) and CPV-isolate B-infected (p) plants*

(*a*)

Genotype	Treatment: Hh	Hp	Ph	Pp	Mean	L.S.D. at 5%
33	44·4	31·1	14·6	13·7	25·9	5·7
43	43·1	38·5	35·2	32·0	37·2	
Mean	43·7	34·8	24·9	22·8	—	—
L.S.D. at 5%	6·2					8·8

(*b*)

Rhizobium source	Plant condition: H	P	Mean	L.S.D. at 5%
h	43·7	24·9	34·3	4·4
p	34·8	22·8	28·8	
Mean	39·2	23·8	—	—
L.S.D. at 5%	4·4			6·2

Table 7. *Analysis of total number and size distribution of nodules produced by healthy and CPV-isolate B-infected cuttings of 2 white clover genotypes, grown in Jensen agar culture and inoculated with w4 cultures of Rhizobium trifolii obtained from healthy and from CPV-isolate B-infected plants*

Source of variation	*N*	Mean square
Genotypes	1	3990·2 *
Replicates	2	49·7
Error A	2	71·8
Size	2	2086·4 ***
Size × genotypes	2	453·4 **
Error B	8	40·3
Treatment		
Plant condition	1	355·6 **
Rhizobium source	1	16·0
Condition × source	1	112·5
Treatment × genotype		
Plant condition	1	128·0
Rhizobium source	1	122·7
Condition × source	1	364·5 **
Treatment × size		
Plant condition	2	534·3 ***
Rhizobium source	2	160·8 *
Condition × source	2	95·5
Treatment × genotype × size		
Plant condition	2	91·8
Rhizobium source	2	54·3
Condition × source	2	212·0 *
Error C	36	46·4

It is evident from the analysis and from Table 6 (*b*), where the two genotypes have been pooled, that although plant condition was the greater source of variation ($P = 0.001$), Rhizobium source also had a major effect on plant growth in a nitrogen-free medium ($P = 0.05$). This is illustrated in Pl. 1, fig. 2. There was no interaction of plant genotype with Rhizobium source, but only a genotype with plant condition which would appear to have been due to the greater effect of the virus on genotype 33 than on 43. There was also no plant condition/Rhizobium source interaction.

Table 8. *Mean number and size distribution of nodules per plant produced by healthy (H) and CPV-isolate B-infected (P) cuttings of 2 white clover genotypes, grown in Jensen agar culture and inoculated with w4 cultures of Rhizobium trifolii obtained from healthy (h) and from CPV-isolate B-infected (p) plants*

(*a*)

	Genotype 33				Genotype 43				
Treatment	Small	Medium	Large	Total*	Small	Medium	Large	Total*	L.S.D. at 5%
Hh	8·6	3·7	8·4	20·7	9·6	5·0	11·2	25·8	6·3*
Hp	3·9	1·6	4·7	10·2	15·9	7·0	6·6	29·5	
Ph	3·6	3·0	0·0	6·6	15·2	5·7	5·1	26·0	
Pp	7·3	2·7	0·0	10·0	15·7	4·3	5·7	25·7	
Mean	5·8	2·7	3·3	11·9†	14·1	5·5	7·1	26·8†	—
L.S.D. at 5%				2·0					3·6

† L.S.D. at 5% for comparing these 2 means = 8·7.

(*b*)

Rhizobium source	Healthy cutting (H)				Infected cuttings (P)				
	Small	Medium	Large	Total*	Small	Medium	Large	Total*	L.S.D. at 5%
h	9·1	4·3	9·8	23·2	9·4	4·3	2·6	16·3	4·5*
p	9·9	4·3	5·6	19·8	11·5	3·5	2·8	17·8	
Mean	9·5	4·3	7·7	21·5†	10·4	3·9	2·7	17·0†	—
L.S.D. at 5%				1·8					2·6

* L.S.D. applies to columns asterisked in respective tables.
† L.S.D. at 5% for comparing these 2 means = 3·0.

The data for nodule number and size distribution 8 weeks after inoculation (Tables 7, 8) show that, as for plant weight, plant condition and Rhizobium source both had a major effect. Inevitably, plant condition exerted the greatest influence on nodulation, but the source of Rhizobium, although not affecting total number, had a significant effect on size. There was, however, no interaction between genotype and either plant condition or Rhizobium source; genotypes appear largely to have influenced nodule size independently of the main effects attributable to either treatment. There was a significant interaction between plant condition and Rhizobium source in relation to genotype. Table 8 (*a*) shows this interaction to have been expressed in the production of small nodules. No such interaction is evident in the data for large nodules where, although a greater number was produced consistently by plant genotype 43, this genotypic effect was independent of either plant condition or

Rhizobium source. Of the two genotypes no. 43 produced a significantly greater number of nodules of all classes.

Table 8 (*b*) compares the effect of plant condition and Rhizobium source on the mean combined number and size of nodules produced by both genotypes. In this experiment, healthy plants unexpectedly produced a significantly greater total number of nodules than did the infected plants. However, this was due entirely to an abundance of large effective nodules. Rhizobium source had no significant effect on the total number of nodules, but healthy cuttings produced a significantly greater number of large ones when inoculated with rhizobia from healthy plants, and CPV infected cuttings produced a significantly greater number of small nodules when inoculated with rhizobia from CPV infected plants. Thus, transfer of formerly effective Rhizobium from CPV-infected plants produced on both healthy and infected cuttings nodulation patterns indicative of a shift towards ineffectiveness.

Possible transfer of virus All plants of genotype 43 from the second cutting experiment, which were transferred from tubes to pots of soil and maintained in the glasshouse, flowered by mid June. None showed symptoms of CPV. No plants of genotype 33 had flowered by this date, neither had 60% of the plants from the seedling experiment. However, the remainder produced normal inflorescences without CPV symptoms. Thus it appears that the rhizobia did not act as a vector of this virus.

DISCUSSION

When inoculated with a normally highly effective strain of Rhizobium, white clover plants infected with clover phyllody virus (CPV) gave a smaller yield than similarly inoculated uninfected plants, and exhibited a partially ineffective type of nodulation. The high correlation between plant weight as a measure of nitrogen fixation and the number of large nodules produced, provides a clear indication that the nodule characters noted (increased time to nodulation, less pigmentation, a generally greater total number of nodules but fewer large ones) are valid criteria of Rhizobium ineffectiveness. Although these results were obtained under the precise but artificial conditions obtaining in test-tube culture, it is reasonable to assume that they would be also applicable to natural field conditions.

Contrary to the contention of Vanderveken (1964), transference of rhizobia from virus-infected to healthy plants, either directly from nodules or after several agar transfers, did not restore full effectiveness. Even on virus-infected plants, nodulation was less effective with Rhizobium from a virus-infected plant than from an uninfected one.

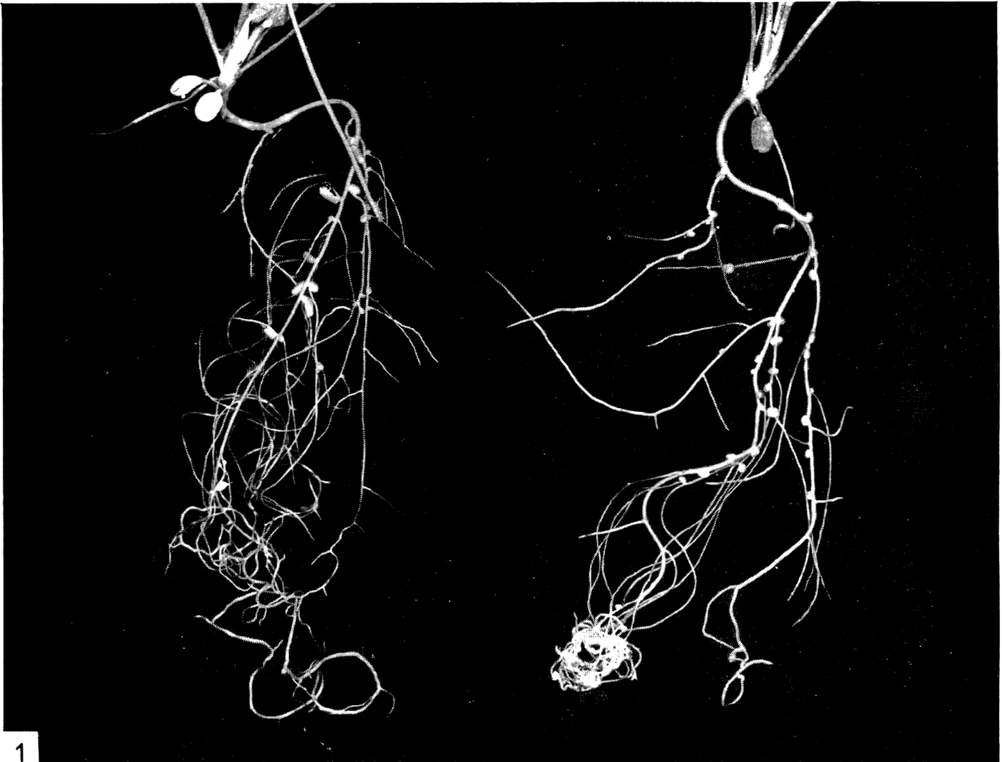
The occasionally significant between-isolate variation, which occurred in experiments with several different virus isolates, suggests that the virulence of a particular virus isolate influences the degree of Rhizobium ineffectiveness, both in terms of the host plant itself and the plant as a source of Rhizobium. Time of sampling, host genotype, and the size of the nodule from which rhizobia were isolated originally, all had a major effect on growth and nodulation. The results obtained for the effect of Rhizobium isolated from nodules of different sizes are contrary to the reports of Holding & King (1963). None of these three factors exerted a major influence on the degree of ineffectiveness induced by CPV, but were not studied in all experiments; hence no firm conclusion can be drawn from this apparently small order of interaction.

particularly for genotype, where the only two genotypes included could not be entirely representative of the range of possible genotypic expression. Size of nodule which serves as a source of Rhizobium showed marked interaction with plant condition, but as this was due entirely to one isolate it cannot be assumed that this was a major factor influencing induced ineffectiveness.

The failure of Rhizobium to transmit CPV parallels the results of Johnson & Jones (1943), who were similarly unsuccessful in transmitting pea enation mosaic, pea severe mosaic and white clover mosaic viruses by this means, except in one unsubstantiated instance. The persistence of this partial ineffectiveness after transfer of rhizobia from CPV infected to healthy plants was clearly not due to transfer of virus, but to some modification of the rhizobia, induced either by virus-infected tissue or by the virus itself. A parallel example, though of opposite effect, is to be found in the change towards greater nodulation potential induced in Rhizobium inoculum following passage through graft and sexual hybrids of *Trifolium ambiguum* with *T. hybridum* (Evans & Jones, 1964). At the present time, the precise mechanism of this induced change is a matter of speculation. Since the rhizobial culture was derived from a single colony it is unlikely to have been due to selection of accumulated mutants, although in view of the technique of isolation used this possibility cannot be excluded entirely. Even in the event that the colony derived from more than one cell, the variation available for such selection would be strictly limited. There remains the possibility of simple adaptation (Cohen & Monod, 1957), or of genetic chromosomal, episomal (Jacob, Schaeffer & Wollman, 1960) or extra-chromosomal (Beale, 1958; Jinks, 1964) change induced either as a direct mutation or by transfer of genetic material from virus to bacterium (Zinder & Lederberg, 1952; Stocker, Zinder & Lederberg, 1953). The latter possibility (a phage-type transductive mechanism) would be unique for such a virus and would require explanation for the apparent failure of ultimate reconstitution of the independent virus. Further, the maintenance in the Rhizobium stock of a high level of adaptive change exemplified by its behaviour after repeated agar transfer precludes the possibility of 'unilinear transmission' (Stocker, 1956) unless a high selective advantage during agar passage is assumed.

Whatever the mechanism of induction, it is evident that Rhizobium is capable of a great deal of adaptive variation, and it is not improbable that this could also be towards greater effectiveness. In addition to virus, host genotype, soil and other nutritional and biochemical factors might well prove capable of inducing such adaptive change. In this connexion the contention of Nutman (1949), that in red clover the cytoplasmic and nuclear gene complex which conditions resistance to nodulation has no adaptive influence on the rhizobia involved, seems worthy of reinvestigation.

In assessing the significance of this indirect effect of CPV (through its influence on nodulation) it is important to consider the possible outcome of competition between fully effective Rhizobium and those induced to a degree of ineffectiveness. If, as is true of certain strains of ineffective Rhizobium (Nicol & Thornton, 1942), those induced to partial ineffectiveness as a result of virus action compete better than fully effective ones for invasion sites on the host roots, then even uninfected plants in a white clover sward infected with CPV might be expected to show a depression in yield as a result of nodulation with rhizobia from infected plants. The magnitude of this effect would be dependent on whether effectiveness can be fully restored following serial passage through healthy plants.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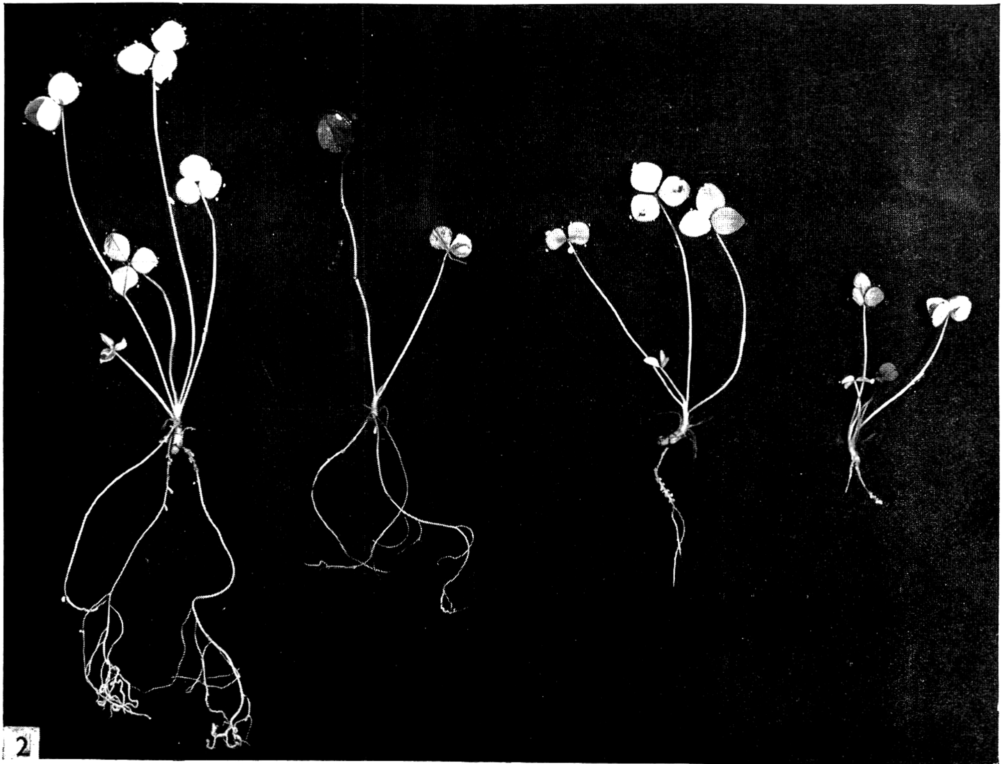
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EXPLANATION OF PLATE

Fig. 1. Root systems of healthy S. 100 white clover seedlings grown in Jensen agar and inoculated with rhizobia from: L., a healthy plant, showing large, obovate, effective nodules confined to the upper root area; R., a CPV-infected plant, showing small, round, ineffective nodules distributed over the entire root system.

Fig. 2. Healthy (H) and CPV-infected (P) cuttings of S. 100 white clover (genotype 33), grown in Jensen agar culture and inoculated with rhizobia from healthy (h) and CPV-infected (p) plants. L. to R.: Hh, Hp, Ph, Pp.

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With 1 *plate*
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The Transmissible Nature of the Genetic Factor in *Escherichia coli* that Controls Haemolysin Production

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SUMMARY

In mixed cultures, a genetic factor (designated Hly factor) responsible for α-haemolysin production in 10 of 53 strains of *Escherichia coli* was transmitted at a relatively high rate to other organisms, including shigellas and salmonellas as well as *E. coli*. Transmission was evidently by conjugation for it was not achieved by bacteria-free culture fluids. Although the factor was not eliminated by acriflavine or ultraviolet irradiation, it was probably a plasmid. Salmonella recipients were very unstable, and during serial subculture in broth the Hly factor was lost from most of these organisms. The factor was easily reintroduced into these segregants, but with difficulty or not at all into the rare *E. coli* organisms which had lost it. Hly factor was transmitted independently of F, R and *col* factors and phages were not involved in its transmission. Strains of *E. coli* K12 F$^+$ into which Hly factor had been introduced became resistant to the F-specific phage. From this it appeared that the factor has the *fi*$^+$ character observed in certain R factors. The illness produced in mice by the intravenous injection of culture fluids of α-haemolytic strains of *E. coli* was shown to be caused by the α-haemolysin itself.

INTRODUCTION

Smith (1963) showed that two haemolysins may be produced by *Escherichia coli*. The most common one, designated α, can be obtained free from the bacteria. Strains of *E. coli* which produce it are frequently found in small numbers in the faeces of human beings, cattle, sheep and pigs. Some of these strains cause clinical disease in the pig. Certain bacterial characters—for instance, drug-resistance (Watanabe, 1963) and colicine production (Fredericq, 1963)—are controlled by genetic elements which are transmissable by conjugation between organisms of the family Enterobacteriaceae, independently of the chromosome. The present paper describes experiments in which it was shown that α-haemolysin production was another character which could be transferred by conjugation. Ten of 53 naturally-occurring α-haemolytic strains of *E. coli* transmitted their haemolytic activity to other bacteria, including strains of the same species, as well as to Salmonella and Shigella strains. The term Hly factor is proposed for the element which is responsible for transmissible α-haemolysin production.

When the Hly factor was transferred to *E. coli* K12 F$^+$, the culture was no longer lysed by the F-specific phage MS2. In this way, the Hly factor behaved like those R factors which have been designated *fi*$^+$ (for fertility inhibition; Watanabe, 1963) because they inhibit the functions of F.

METHODS

The transfer of the ability to produce α-haemolysin. Nutrient broth (Oxoid no. 2) in 10 ml. amounts was seeded with 0·02 ml. of a 24 hr broth culture of a haemolytic strain of *Escherichia coli* and with 0·02 ml. of a similar culture of a non-haemolytic strain. On occasion, the relative sizes of the inocula were varied. In each experiment the prospective recipient non-haemolytic strain was a mutant resistant to one of the drugs ampicillin, streptomycin or nalidixic acid to which the haemolytic strain was sensitive. The mixed cultures were incubated at 37° for 24 hr and then inoculated so as to yield as many as possible well-isolated colonies on 'washed blood' agar containing 20–30 μg. drug/ml. The plates were incubated at 37° for 24 hr and then examined for haemolytic colonies. When any were present, one or two were picked and purified by plating twice on 'washed blood' agar and then examined serologically to confirm that they were colonies of the recipient strain. Auxotrophic mutants of *E. coli* strain K12 were used as recipients in some experiments; here, the bacteria were identified by their growth requirements. In most cases the final culture was examined to determine that the haemolysis produced was due to α-haemolysin (Smith, 1963). In all mixed culture experiments, controls, consisting of nutrient broth that had been inoculated with the recipient strain only, were also tested.

The fi^+ character of Hly factors. This was tested after transfer to F^+ strains of *Escherichia coli* K12, by examining the cultures for visible lysis by phage MS2 (Davis, Strauss & Sinsheimer, 1961). This has been shown to be a valid test for the *fi* character (Meynell & Datta, 1966).

'Washed blood' agar. This consisted of nutrient agar containing washed ox red cells in a concentration equivalent to that in 10% (v/v) ox blood.

The identification of α-haemolysin-producing strains, the titration of cell-free α-haemolysin, the preparation of antisera to haemolysin and the performance of plate antihaemolysin tests were done by the methods of Smith (1963).

RESULTS

The incidence of transmissible haemolysin factor (Hly factor) amongst α-haemolytic strains of Escherichia coli

The α-haemolytic activity of 53 epidemiologically unrelated strains of *Escherichia coli* belonging to 11 different serotypes and pathogenic for pigs was tested for transmissibility. In 10 of the 53 strains the haemolysin was found to be transmissible; despite repeated tests the α-haemolysin of the remaining 43 strains was never shown to be so. Of the 10 positive strains, 7 possessed the antigenic formula O138:K81 and one the antigenic formula O147:K89,88ac. The other two strains belonged to the Abbotstown type, OA1:KA1,88ac, a type that has recently been found by Mr E. J. Sweeney and others (unpublished) to be incriminated in some outbreaks of diarrhoea in pigs; this type has not yet been given an internationally accepted formula. Of the 43 α-haemolytic strains which did not transmit their haemolytic activity 8 were of the antigenic type O138:K81, 2 of the type O147:K89,88ac and 13 of the Abbotstown type.

Most of the non-haemolytic strains used in the mixed culture experiments were able to receive the Hly factor from a donor strain. For example, the Hly factor from

one donor strain, P 233, of the Abbotstown type, was transmitted to 15 of 18 non-haemolytic strains of various antigenic formulae; the remaining 3 strains did not act as recipients for any of the Hly factors tested.

Although variation in the rate of transmission was sometimes noted, from donor to donor and from recipient to recipient, the rate was usually high. Frequently 10–20 % of the colonies of the recipient strain which grew on the drug-containing washed blood agar plate inoculated with the 24 hr mixed culture of recipient + donor strain were haemolytic. A typical result of such an experiment is illustrated in Pl. 1, fig. 1.

The Hly factor was transmitted at the same rate to three different variants of *Escherichia coli* K12, irrespective of whether they were F^+ or F^-. The transfer rate was in the region of 10–20 %.

The presence or absence of R factors (Watanabe, 1963) did not influence the ability of a strain to act as a donor or as a recipient for Hly factor; 5 of the 10 wild strains from which haemolytic activity was transmitted, and 6 of the 43 strains from which it was not transmitted, contained R factors. The presence or absence of Hly factors did not interfere with the ability of a strain to act as a donor or recipient of R factors. In 21 mixed-culture experiments, Hly factor was transmitted from one or other of 6 Hly factor-containing and R factor-containing strains to one or other of 16 recipient strains. Although these experiments were designed to detect transmission of Hly factor, it was noted that R factors also had been transmitted in four of these experiments. Strain P 233, which contained both the Hly factor and an R factor which conferred neomycin-resistance, was grown with three different non-haemolytic neomycin-sensitive K12 strains, two F^+ and one F^-. The results with all three mixtures were similar. Approximately 10 % of the K12 organisms were now haemolytic. The proportion of neomycin-resistant organisms was much lower, about 0·01 %, about half of which were haemolytic. The F^+ strains into which neomycin resistance only had been transmitted retained their sensitivity to F-specific phage. The R factor was, therefore, an fi^- factor. However, when haemolytic activity only or haemolytic activity + neomycin resistance had been transmitted, the F^+ cultures became resistant to F phage. Resistance to F phage was a feature of all F^+ strains into which Hly had been introduced. During one experiment in which Hly factor was transmitted from strain P233 to strain K12 F^+, about 15 % of the haemolytic colonies had also acquired the K88 antigen from strain P233, thus confirming the observation of Ørskov & Ørskov (1966) that the factor which controls this K88 antigen is transmissible in mixed culture.

The transmission of Hly factor from Escherichia coli to other bacterial species

By using several different donors, attempts were made to transmit Hly factor from *Escherichia coli* to four strains of *Shigella sonnei* and to one strain each of *Shigella flexneri* 2a, 3a and 4a, *Salmonella typhimurium*, *S. dublin*, *S. cholerae-suis*, *Actinobacillus lignieresi*, *Chromobacterium violaceum*, *Klebsiella aerogenes*, *K. pneumoniae*, *Pasteurella pseudotuberculosis*, *P. septica*, *Proteus morgani*, *Pseudomonas aeruginosa*, a *Bacillus* sp., *Staphylococcus aureus* and *Streptococcus faecalis*. Transmission was achieved to 3 of the 4 *Shigella sonnei* strains, the 3 *Shigella flexneri* strains and the 3 Salmonella strains. Despite repeated attempts it was impossible to transmit Hly factor to any of the other bacteria. The transfer rate of Hly factor to the Salmonella

and Shigella strains (0·5–5 %) was usually lower than that observed when *E. coli* strains were used as recipients. The Hly factor was easily retransmitted from these Salmonella and Shigella strains to non-haemolytic strains of *E. coli*.

The nature of the haemolysin produced by Hly⁺ and other strains of Escherichia coli

Since only 10 of 53 α-haemolytic strains of *Escherichia coli* were shown to transmit their haemolytic activity, antisera were prepared in rabbits against the haemolysins produced by a transmitting and a non-transmitting strain. Plate anti-haemolysin tests were then made against six wild Hly^+ strains and six wild strains whose haemolytic activity was not transmitted. The results did not reveal any difference between the haemolysin of the two groups.

Very little difference was noted in yield of α-haemolysin between wild strains of *Escherichia coli* whose haemolytic activity was not transmitted and wild strains shown to contain Hly factor, and strains of *E. coli*, Salmonella and Shigella into which Hly factor had been introduced in the laboratory.

The significance of culture filtrates, phages and colicines in Hly factor mediation

Culture filtrates. When the α-haemolytic strain P233 was grown in mixed culture with one or other of two different non-haemolytic strains of *Escherichia coli*, approximately 10 % of the organisms of these two strains accepted the Hly factor of strain P233. When the experiments were repeated with 6 ml. of a filtrate (which had been passed through a membrane of average pore diameter 250 mμ) of a broth culture of strain P233 in place of living organisms, no haemolytic organisms were isolated.

Phages. None of 16 Hly^+ strains of *Escherichia coli*, comprising 6 naturally occurring and 10 to which Hly factors had been transferred in the laboratory, could be shown to be lysogenic when tested against 9 strains of *E. coli* and one strain each of *Salmonella dublin*, *S. choleraesuis* and *Shigella sonnei*, strains that had been shown to act as recipients of Hly factor.

Colicines. The two groups of strains used in the previous experiment were also examined to see whether any of the Hly-factor donors produced a colicine. Only 2 of the 16 donors were found to be colicinogenic, the colicines produced by both being active against the same 5 strains of *Escherichia coli*.

In some mixed cultures Hly factor was transmitted from a strain that produced colicine active against the recipient strain; in such instances the colicinogeny was not transmitted at the same time as the Hly factor. In some, but not all, cases in which a non-haemolytic strain of *Escherichia coli* did not act as a recipient of Hly factor this failure appeared to be associated with the fact that the strain produced a powerful colicine active against the Hly^+ donor itself.

The stability of the Hly factor in different bacteria

Strains known to contain Hly factor were passaged daily for 20 days in nutrient broth at 37° by using small inocula transferred with the tip of a platinum wire. After incubation, each subculture was inoculated on washed blood agar to determine whether it contained any organisms that had lost the Hly factor.

Escherichia coli. The Hly factors were relatively stable in this species, for Hly^-

segregant colonies were only occasionally seen, and did not become more numerous in the later subcultures. For instance, of six wild Hly+ strains, one yielded one or two non-haemolytic colonies in four subcultures and two yielded one non-haemolytic colony once; only haemolytic colonies were found with the other three strains. Seven strains into which Hly factor had been introduced in the laboratory were subcultured. Apart from one strain which yielded approximately one non-haemolytic colony to every 40 haemolytic colonies at each subculture, all the other strains consistently yielded only haemolytic colonies.

Shigella species. The three strains of *Shigella sonnei* into which Hly factor had been introduced in the laboratory were subcultured; one always yielded haemolytic colonies only; another gave one or two non-haemolytic organisms following a few subcultures. By contrast, the third strain was much less stable, for an increasing number of non-haemolytic colonies were found following each subculture. After the fifth subculture, the ratio of non-haemolytic to haemolytic colonies was 40:1. The ratio increased to about 50:1 at the eleventh passage, and remained the same thereafter. To see whether the stability of the Hly factor in this strain would increase with time since infection, this strain was also submitted to serial subculture on washed blood agar plates, the inoculum for each subculture consisting of a suspension of a single haemolytic colony from the previous subculture. The ratio of non-haemolytic to haemolytic colonies on the washed blood agar plates fluctuated considerably but did not show any trend either towards an increase or a decrease. During the first ten subcultures it varied from 1:1 to 40:1. From the tenth to the fifteenth subculture it was approximately 30:1 and from the sixteenth to the twentieth approximately 200:1. Subculture in broth of the three *Shigella flexneri* strains into which Hly factors had been introduced in the laboratory resulted in the isolation of haemolytic organisms only.

Salmonella species. Instability of the Hly factor was a pronounced feature of *Salmonella typhimurium*, *S. dublin* and *S. choleraesuis*, into which it had been introduced in the laboratory. After each subculture in broth an increasing number of non-haemolytic organisms were isolated. By the fifth subculture the cultures appeared to consist only of non-haemolytic organisms.

By the technique used in studying the unstable strain of *Shigella sonnei*, the three haemolytic Salmonella strains were submitted to serial subculture on washed blood agar. The relative numbers of non-haemolytic and haemolytic colonies fluctuated considerably from subculture to subculture. In these studies the strain of *Salmonella dublin* was the most stable, the ratio of non-haemolytic to haemolytic colonies isolated varying from 2:1 to 1:10, usually the latter. The ratio at each of the 20 examinations in the case of the *Salmonella typhimurium* and *S. choleraesuis* strains ranged from 20:1 to 1:10, the latter ratio being more commonly found towards the end of the subcultivation period. However, when a single haemolytic colony from the twentieth serial subculture of both the *S. typhimurium* and *S. choleraesuis* strains was submitted to subcultivation in broth, all the colonies which grew after the third passage were non-haemolytic. The results of inoculating a washed blood agar plate with a suspension of a haemolytic colony of *S. typhimurium*, *S. dublin*, *Shigella flexneri* and the unstable *S. sonnei* strain is illustrated in Pl. 1, fig. 2.

The effect of acriflavine and ultraviolet radiation on the stability of Escherichia coli strains containing Hly factor

Eight Hly+ strains of *Escherichia coli*, six wild and two laboratory-prepared, were grown at 37° for 24 hr in nutrient broth containing concentrations of acriflavine from those that had no apparent effect on growth to those which almost completely inhibited multiplication. When these cultures were inoculated on washed blood agar they yielded either no non-haemolytic colonies or, in the case of some of the cultures of two laboratory strains, only one or two, certainly no more than the number obtained from control cultures not containing acriflavine.

Suspensions of two of the strains were also ultraviolet-irradiated before being inoculated into broth containing acriflavine. Different exposure times were used, from one which caused no lethal effect to one which killed practically all the organisms. No non-haemolytic organisms were isolated.

Immunity to reintroduction of Hly factor

As mentioned previously, a very small number of non-haemolytic organisms were occasionally found during passage of four Hly+ strains of *Escherichia coli.* Attempt were made to reintroduce Hly factor into cultures of these non-haemolytic organisms. Five Hly+ strains of *E. coli* were used as prospective donors. Three of these prospective donors were the parent strains from which three of the non-haemolytic segregants had been derived. Despite repeated attempts, it was impossible to reintroduce Hly factor into one of the four non-haemolytic cultures. The factor was reintroduced into the other three from only one of the five donors at a very low rate and then not at every attempt. By contrast, Hly factors were easily reintroduced into the non-haemolytic organisms obtained from Hly+ Strains of *Salmonella dublin* and *S. choleraesuis.* Similar results were obtained with non-haemolytic organisms derived from the unstable Hly+ strain of *Shigella sonnei.*

The toxic effect in mice of haemolysin-containing culture fluids of strains of Escherichia coli to which Hly factor had been transmitted in the laboratory

Mice injected intravenously with 0·6 ml. of haemolysin-containing bacteria-free fluids from cultures of nine strains of *Escherichia coli* to which Hly factor had been transferred in the laboratory became ill within 2 hr. They were dull, their respirations were more rapid and deeper and they had severe haemoglobinuria, signs similar to those previously observed in mice injected with culture fluids of α-haemolytic strains of *E. coli* (Smith, 1963). Mice injected with similar amounts of culture fluid of the strains before Hly factor had been introduced into them showed little sign of ill-health.

The influence of Hly factor on the pathogenicity of Escherichia coli for mice

Hly factor did not appear to influence the pathogenicity for mice of *Escherichia coli* organisms given by the intraperitoneal route. For example, 3 groups of 15 mice were injected intraperitoneally with about 10^8 viable organisms of either a non-haemolytic strain of *E. coli* O18:K? suspended in phosphate buffer (pH 7·0), or of two cultures of this strain after the introduction of Hly factor from two serologically-different α-haemolytic strains of *E. coli.* All three groups of mice became very ill and about one-third of those in each group died, all deaths occurring within 24 hr.

The influence of Hly factor on the pathogenicity of salmonella for mice

Five mice were injected subcutaneously with 2000–3000 viable organisms of a 24 hr broth culture of the non-haemolytic strain of *Salmonella typhimurium* referred to previously, and five mice with a similar dose prepared from this strain after the introduction of Hly factor. Identical experiments were made with the *S. dublin* and *S. choleraesuis* strains before and after the introduction of Hly factor. None of the 30 mice died. When they were killed 12 days after the beginning of the experiment there was no difference in the incidence and severity of the liver lesions in the mice which had been given haemolytic organisms and in those given non-haemolytic organisms.

In another experiment, 3 groups, each of 10 mice, were injected subcutaneously with one or other of the three haemolytic Salmonella strains. One mouse from each group was killed every day and its liver inoculated on to washed blood agar to determine the proportion of haemolytic and non-haemolytic salmonellas present. The highest ratio of haemolytic to non-haemolytic organisms of *S. typhimurium* found was 1:20; on several occasions only one or two haemolytic colonies amongst a mass of non-haemolytic colonies were seen on the washed blood agar plates. The haemolytic:non-haemolytic ratio found at all the daily examinations of the mice inoculated with haemolytic *S. choleraesuis* was about 1:20. In the first few examinations of the mice inoculated with *S. dublin* the ratio was 1:2–5; at subsequent examinations a progressive decrease in the relative numbers of haemolytic organisms was noted; at the final examination it was about 1:40.

DISCUSSION

The results indicate that the ability to produce α-haemolysin can be transmitted from some strains of *Escherichia coli* and that transmission when it occurs is by conjugation. This is analogous to what has been found with R factors, *col* factors and F (Hayes, 1964; Datta, 1965). The evidence so far suggests that transmission may be considered to result from the presence of a supernumerary extrachromosomal genetic element, or plasmid, carrying a gene which determines the production of the haemolysin, in the same way as R factors carry the genes which confer drug resistance (Watanabe, 1963). The name 'Hly factor' is proposed for this element.

No exclusion of the kind described by Scaife & Gross (1962) for F could be demonstrated between the Hly factor and either F or R factors; they could be carried together in the same bacterium. Nor could exclusion be demonstrated between Hly and *col* factors. When an R factor was present with Hly there was evidently no association between them, for they were transmitted independently of each other. In one R^+ strain of *E. coli*, the particular R factor was apparently fi^- (Watanabe *et al.* 1964) for K 12 F^+ strains into which it was introduced remained sensitive to the F-specific phage. The Hly factor which this strain also contained, like the Hly factors in all the other strains tested, resembled the fi^+ R factors of Watanabe *et al.* (1964) in rendering K 12 F^+ strains resistant to the F specific phage.

The stability of Hly was high in most strains of *Escherichia coli* and Shigella and low in the Salmonella strains. Variability in stability is also a feature of R factors (Watanabe, 1963). Hly factor differed from F in not being eliminated by treatment

with acriflavine or ultraviolet radiation, but many other plasmids, e.g. some R factors and *col* factors, are not eliminated in this way by either of these treatments.

Despite the fact that the haemolytic activity of all the strains of *Escherichia coli* examined appeared to be identical, it was not possible to demonstrate transmission from most of the strains. Several reasons may be put forward to account for this. Perhaps in these strains the genes responsible for haemolysin production are integrated in the chromosome. On the other hand, plasmids may be responsible, but ones which have never had or have lost conjugation-factor activity, or which are defective in this respect (Cuzin & Jacob, 1965). Alternatively, the plasmids may simply be repressed like R factors (Meynell & Datta, 1966); our techniques were such that low frequencies of transfer would not be detected.

Not only did the Hly+ Salmonella and *Escherichia coli* strains differ greatly in stability but Hly was easily reintroduced into Salmonella organisms, but not into *E. coli* organisms, which had lost it. If the Hly system resembles the other systems previously referred to, then it probably consists of genes which govern the actual production of haemolysin and other genes responsible for the conjugation and transmission of these genes to other organisms. If this be so, it is possible that the Salmonella segregants had lost the complete genetic element whereas the *E. coli* organisms which lost the haemolytic activity still retained the part concerned with exclusion (Scaife & Gross, 1962). It is possible that the few wild strains of *E. coli* into which we failed to introduce Hly excluded it for the same reason.

It would appear then that haemolytic activity is yet another transmissible character to add to the list of R factors, *col* factors and the antigen discovered by Ørskov & Ørskov (1966). One might imagine that characters transmitted in this way should give some selective advantage to the organisms and it is easy to see, for example, what the advantage might be with R factors. The advantage in the present case might reflect pathogenicity, evidenced by experiments in which the culture fluids of strains of *Escherichia coli* before and after the introduction of Hly were injected intravenously into mice. Although no difference was observed in mice in experimental infections with whole organisms, the haemolysin itself was toxic. The great majority of strains of *E. coli* causing disease in pigs are α-haemolytic.

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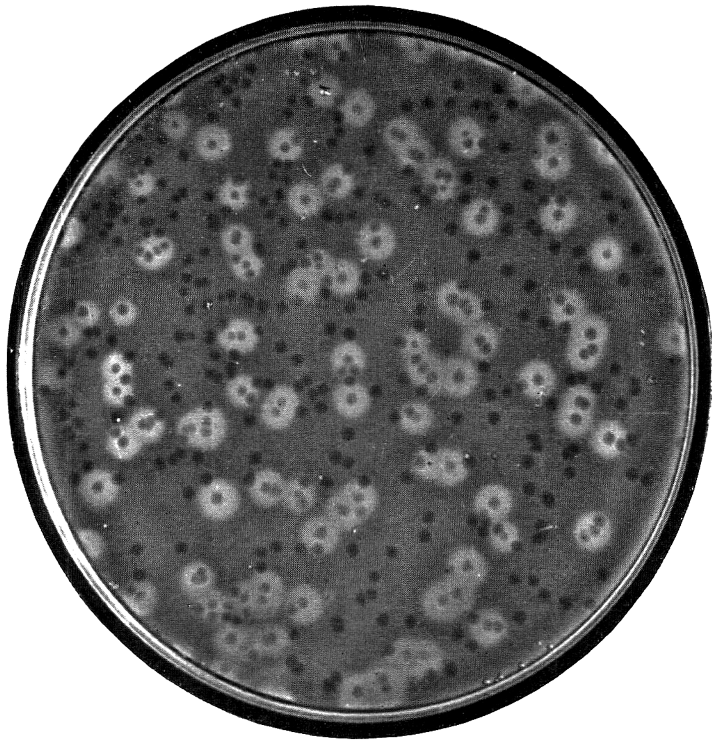

Fig. 1

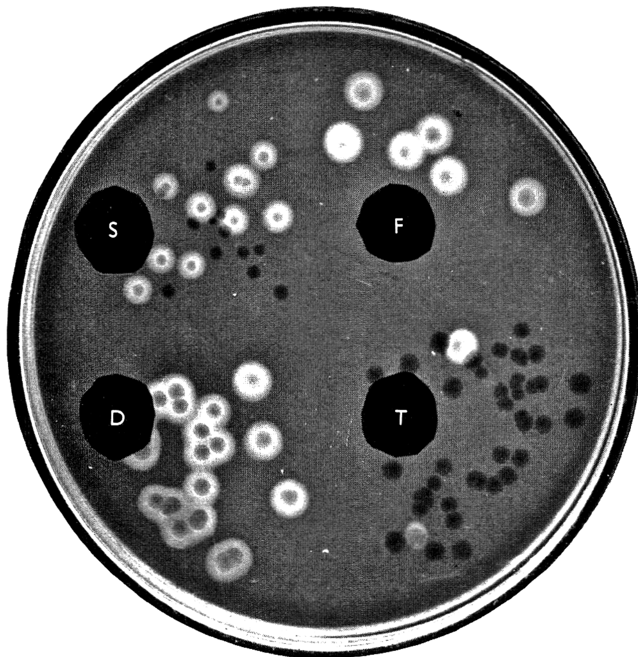

Fig. 2

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EXPLANATION OF PLATE

Plate 1

Fig. 1. Result of a mixed culture experiment. The mixed culture of an α-haemolytic donor strain of *Escherichia coli* and a non-haemolytic recipient strain of *E. coli* has been inoculated on to a washed blood agar plate containing an antibiotic which has completely suppressed the growth of the donor strain. The colonies are those of the antibiotic-resistant recipient strain; about 10% of them are now haemolytic. × 1½.

Fig. 2. Strains of shigellas and salmonellas into which Hly factor has been introduced. The plate has been lightly inoculated with suspensions of haemolytic colonies of *Shigella sonnei* (S), *S. flexneri* (F), *Salmonella typhimurium* (T) and *S. dublin* (D). The instability of the Hly factor in *Shigella sonnei* and *Salmonella typhimurium* strains is apparent. × 1⅓.