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Ganglioside as a Prophylactic Agent in Experimental Tetanus in Mice

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SUMMARY

The symptoms of tetanus in mice, resulting from intramuscular injection of either purified tetanus toxin or vegetative bacilli of *Clostridium tetani*, can be partially prevented if the mice are injected at the same time, or a few hours before or afterwards, with a preparation of mixed gangliosides or with a suspension of ganglioside/cerebroside complex. Injection at the same site as the toxin injection is most effective, although intravenous injection of ganglioside (but not of the complex) also has some action; 0·5 mg. of ganglioside when complexed with 1·5 mg. cerebroside is as effective in protection as 5 mg. ganglioside alone. Protection by complexes containing different proportions of ganglioside reflects their ability to fix tetanus toxin *in vitro*. It is tentatively suggested that injection of ganglioside/cerebroside complex at a site of injury might be of prophylactic value in human tetanus.

INTRODUCTION

Tetanus toxin combines with nervous tissue (Wassermann & Takaki, 1898), and the receptor substance for the toxin involves gangliosides (van Heyningen, 1959; van Heyningen & Miller, 1961; van Heyningen & Mellanby, 1968). It is not known whether the combination of tetanus toxin with ganglioside plays an essential part in the development of tetanus symptoms. However, it seemed possible that injection of ganglioside might affect the course of the disease. In the present work, experimental tetanus was induced in mice by injecting either purified toxin, or whole vegetative bacilli of *Clostridium tetani*. The effect of injecting ganglioside alone, or ganglioside complexed with cerebroside, at different times relative to the injection of toxin and by different routes, on the subsequent development of tetanus symptoms was examined. Hence it has been possible to investigate the prophylactic effect of such injections in experimentally induced tetanus in mice.

METHODS

Tetanus toxin was kindly provided by Dr R. O. Thomson (Wellcome Research Laboratories). Dilutions of toxin were made in 0·1 M-phosphate (pH 7·0) containing 0·2% gelatin (gelatin-buffer). Mixed gangliosides were prepared by the method described by Mellanby, Pope & Ambache (1968). Cerebroside was prepared by the method of Klenk & Leupold (1944). Ganglioside/cerebroside complexes were prepared as described by van Heyningen & Mellanby (1968). Sialic acid was determined by the resorcinol + hydrochloric acid method of Svennerholm (1957).

The strain of *Clostridium tetani* used was kindly provided by the Wellcome Research Laboratories. It was inoculated into sloppy agar Mueller medium (Mueller & Miller, 1954). For the experiments, 250 ml. Mueller medium was inoculated with 0·5 ml. of an overnight culture in the sloppy agar, and incubated for 20 hr at 37°. The organisms were collected by centrifugation at 9000 *g* for 15 min., washed with 250 ml. 0·9 % NaCl and then resuspended in a suitable volume of 0·9 % NaCl. Dilutions were made in 0·9 % NaCl and these were injected into mice.

Table 1. *Values assigned to symptoms of local tetanus induced in mice by injecting toxin intramuscularly into a hind limb*

Symptom	Value assigned
No symptoms	0
Slight stiffness in injected limb visible only when mouse suspended by tail	1
An obvious limp in injected limb but it is still used effectively in walking	2
Injected limb still movable, but not functional	3
Injected limb rigid and even the toes immovable	4
Animal convulsing and generally very ill	5
Dead	6

The symptoms of tetanus in mice were assessed on a numerical scale (Table 1). The symptoms in each group of mice under observation were assessed daily, for 7 days, after injection of tetanus toxin or vegetative bacilli of *Clostridium tetani*. In each group, the mean of the values assigned to the symptoms was calculated. In Fig. 1 the mean symptom (and the standard deviation of each point) in a group of 20 mice injected with 2 LD 50 of tetanus toxin is plotted against the time after injection. Figure 1 shows, incidentally, that the standard deviation in a group of injected mice was greater when the animals were near death. Death is affected by factors which do not influence the symptoms of intoxication—whether an animal dies at symptom 4 may depend on such extraneous factors as cannibalism (it was not feasible for us to use single-mouse cages). Therefore the intermediate effects are more meaningful than the final effects—which are the only ones observed in the customary determinations of either lethal dose or survival time.

RESULTS

Preliminary experiments indicated that the severity of tetanus symptoms in mice after injection with tetanus toxin was decreased when ganglioside had been injected some hours before the toxin. Table 2 shows the results of an experiment in which the effect of injecting ganglioside at different times and by different routes was tested on the subsequent development of tetanus symptoms (after injection of tetanus toxin). The experiment was done with 20 groups of 10 mice. The four routes chosen for injection of ganglioside were: intramuscularly in the same leg which was subsequently injected with toxin; intramuscularly in the opposite hind leg; intravenously into the tail vein; intraperitoneally. Twenty-four hr before injection of toxin (2 LD 50) each of four groups of mice was injected with ganglioside by one of these four routes. Similarly, injections were done in three more sets of four groups of 10 mice, with the ganglioside injected 6 hr or 2 hr before the toxin, or at the same time (that is, a few seconds

afterwards). A further four groups of 10 mice were injected with gelatin-buffer by one of each of the four routes, 2 hr before injection of toxin. The mice were examined daily for 7 days after toxin injection and their tetanus symptoms assessed according to the scale in Table 1. Table 2 shows that the mice which were given ganglioside at the same time as toxin, at the same site of injection, were protected against all but the mild symptoms of tetanus. Thus, while on the 7th day after injection of toxin all the control mice (injected with toxin and gelatin-buffer) were dead, the ganglioside-injected mice showed only a limp (mean symptom 2·6+0·5). Ganglioside given before the toxin at the same site as the toxin was less effective in protecting against tetanus the earlier it was given. When ganglioside was injected by other routes it was much

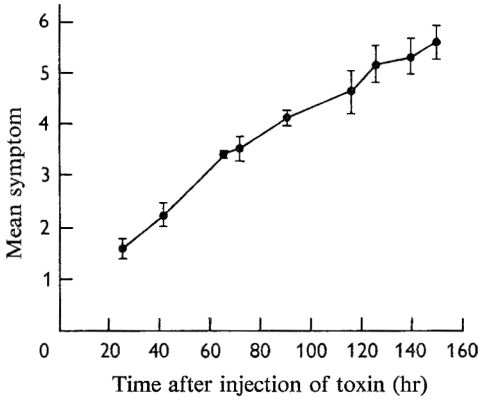

Fig. 1

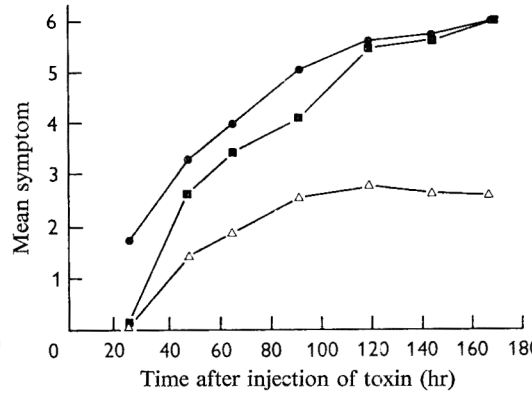

Fig. 2

Fig. 1. The progression of tetanus symptoms in a group of 20 mice injected with 2 LD 50 tetanus toxin. (See Table 1 for assessment of symptoms. The vertical lines indicate the standard deviations of the values.)

Fig. 2. Protection of mice against 5 LD 50 of tetanus toxin with ganglioside/cerebroside complex (25 % ganglioside). The values are the means of the symptoms in each group of 20 mice (see Table 1). All the injections were intramuscular in the same hind limb. ●, Gelatin-buffer; ■, 0·5 mg. ganglioside; △, 2 mg. ganglioside/cerebroside complex.

less effective, and the slight degree of protection it afforded was the same whether it was injected at the same time as the toxin or 2 or 6 hr before. Ganglioside had no effect when injected (by any route) 24 hr before the toxin. Intravenous injection of an amount of ganglioside which was only one quarter of that injected by the other routes had as much protective effect against tetanus toxin as the larger dose injected intraperitoneally or intramuscularly in the opposite leg. (This smaller dose was used for intravenous injection because the larger dose when injected intravenously was sometimes lethal to mice suffering from tetanus.)

The observation that the protective effect of the ganglioside was greatest when the ganglioside and toxin were injected at the same site in the animal, and at the same time, suggested that the protection might be due to fixation or inactivation (see van Heyningen, 1961) of the toxin by the ganglioside at the site of injection. It had previously been found that at low concentrations (a few LD 50/ml.), tetanus toxin was fixed by complexes of ganglioside with cerebroside (Mellanby & van Heyningen, 1965; van Heyningen & Mellanby, 1968), the optimal proportion of ganglioside being about

Table 2. *The effect of injecting ganglioside at different times and by different routes on the subsequent development of symptoms after intramuscular injection of tetanus toxin*

The figures are the means of the value ascribed to the tetanus symptom (see Table 1) ±the standard deviation in the group under consideration; the figures in parentheses denote the number of animals in the group.

Time (hr) before toxin that ganglioside was injected	Route of ganglioside injection	Day 3	Day 5	Day 7
24	Intraperitoneal	4·0±0·67 (10)	5·5±0·72 (10)	6·0±0 (10)
	Intravenous	4·2±0·78 (10)	5·3±0·48 (10)	6·0±0 (10)
	Intramuscular in same leg as toxin	3·5±0·53 (10)	4·8±0·79 (10)	6·0±0 (10)
	Intramuscular in opposite leg to toxin	4·2±0·42 (10)	5·3±0·48 (10)	6·0±0 (10)
6	Intraperitoneal	3·5±0·56 (9)	4·3±1·38 (9)	4·9±1·05 (9)
	Intravenous	3·3±0·48 (10)	3·8±0·21 (10)	4·4±0·89 (10)
	Intramuscular in same leg as toxin	2·4±0·52 (10)	3·5±0·71 (10)	4·5±1·18 (10)
	Intramuscular in opposite leg to toxin	3·3±0·48 (10)	4·3±0·48 (10)	4·7±0·68 (10)
2	Intraperitoneal	3·6±0·49 (10)	4·3±0·45 (10)	4·7±1·16 (10)
	Intravenous	3·1±0·57 (10)	3·9±0·78 (10)	4·3±0·48 (10)
	Intrasmuscular in same leg as toxin	1·8±0·21 (10)	3·0±0 (10)	3·3±0·67 (10)
	Intramuscular in opposite leg to toxin	3·5±0·53 (10)	4·4±0·70 (10)	4·6±0·85 (10)
0	Intraperitoneal	4·0±0 (10)	4·1±0·31 (10)	4·8±1·03 (10)
	Intravenous	3·6±0·53 (9)	4·0±0·87 (9)	4·5±0·90 (9)
	Intramuscular in same leg as toxin	1·7±0·48 (10)	2·3±0·48 (10)	2·6±0·52 (10)
	Intramuscular in opposite leg to toxin	4·2±0·21 (10)	4·3±0·67 (10)	5·3±0·48 (10)
Gelatin-buffer injected 2 hr before toxin	Intraperitoneal	4·1±0·74 (10)	5·2±0·84 (10)	6·0±0 (10)
	Intravenous	4·0±0 (10)	5·5±0·85 (10)	6·0±0 (10)
	Intramuscular in same leg as toxin	4·0±0 (10)	5·0±0·81 (10)	6·0±0 (10)
	Intramuscular in opposite leg to toxin	4·1±0·31 (10)	5·4±0·82 (10)	6·0±0 (10)

25 %. If fixation of tetanus toxin by injected ganglioside were the reason for the protective effect of ganglioside, then it might be expected that a complex of ganglioside and cerebroside would also protect against tetanus toxin. Figure 2 illustrates the results of an experiment in which the protective effect of 0·5 mg. ganglioside alone was compared with the protection afforded by 0·5 mg. ganglioside complexed with 1·5 mg. cerebroside. Three groups of 20 mice were used, and 5 hr before the injection of 5 LD 50 of tetanus toxin: the first group was injected with 0·5 ml. gelatin-buffer only, the second group with 0·5 ml. gelatin-buffer containing 2 mg. ganglioside/cerebroside complex (25 % ganglioside) and the third group with 0·5 ml. gelatin-buffer containing 0·5 mg. ganglioside. The mean of the values ascribed to the symptoms

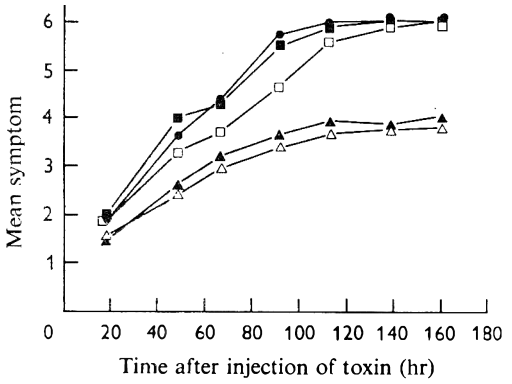

Fig. 3

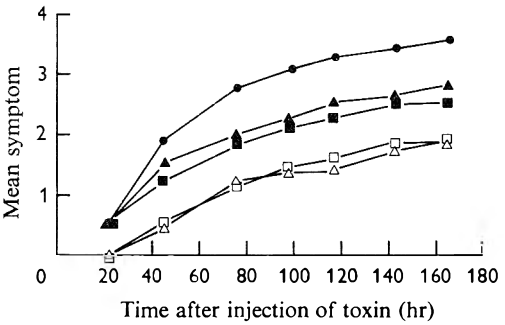

Fig. 4

Fig. 3. Protection of mice against 20 LD 50 of tetanus toxin with ganglioside/cerebroside complexes containing different proportions of ganglioside. The values are the means the symptoms in each group of 20 mice (see Table 1). All the injections were intramuscular in the same hind limb. ●, Gelatin-buffer; ■, cerebroside alone; □, ganglioside/cerebroside (50 % ganglioside); ▲, ganglioside/cerebroside (25% ganglioside); △, ganglioside/cerebroside (5% ganglioside).

Fig. 4. A comparison of the protective effect of ganglioside and ganglioside/cerebroside complex injected 5 hr before or 5 hr after a challenge dose of 0·5 LD 50 tetanus toxin. The values are the means of the symptoms in each group of 20 mice (see Table 1). All the injections were intramuscular in the same hind limb. ●, Gelatin-buffer 5 hr before or 5 hr after; ▲, 2 mg. ganglioside/cerebroside complex 5 hr after; ■, 5 mg. ganglioside 5 hr after; △, 2 mg. ganglioside/cerebroside complex 5 hr before; □, 5 mg. ganglioside 5 hr before.

are plotted against the time after injection of the toxin. It can be seen that the ganglioside/cerebroside complex prevented the development of the severe symptoms of tetanus (the symptoms did not proceed beyond stage 3), whereas 0·5 mg. ganglioside had little effect on the progress of the symptoms. This observation was repeated in further experiments and it was also found that 5 mg. ganglioside alone was required to produce the same protection as 0·5 mg. of ganglioside in a ganglioside/cerebroside complex (see Fig. 4) and it was shown that cerebroside alone had no protective effect.

It had been shown previously that the complex of ganglioside and cerebroside containing 25 % ganglioside was more effective (per unit weight of complex) in fixing tetanus toxin than a complex containing either 5 or 50 % ganglioside. The effectiveness of these complexes in protecting against 5 LD 50 of tetanus toxin injected 5 hr later was compared in the experiment illustrated in Fig. 3. In this experiment, equal amounts of ganglioside

were injected in each case—thus with 50 % ganglioside, 1 mg. complex was used; with 25 % ganglioside, 2 mg. complex; and with 5 % ganglioside, 10 mg. complex. It can be seen (Fig. 3) that the complex containing 50 % ganglioside did not protect the mice against tetanus toxin, while the complexes containing 5 and 25 % prevented death. Although these two complexes were equally effective in protection per unit weight of ganglioside, the complex containing 25 % ganglioside was five times more effective per unit weight of complex. In a further experiment ganglioside/cerebroside complexes did not protect against tetanus toxin when the complex was injected intravenously instead of intramuscularly.

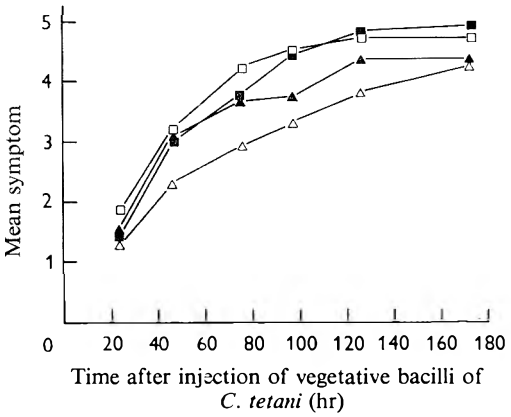

Fig. 5

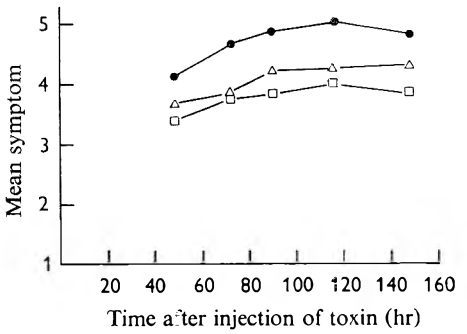

Fig. 6

Fig. 5. A comparison of the effectiveness of intramuscular and intravenous ganglioside in protecting mice against tetanus resulting from the injection of vegetative bacilli of *Clostridium tetani* 5 hr before. The values are the means of the symptoms in each group of 20 mice (see Table 1). The vegetative bacilli of *C. tetani* were injected intramuscularly in the hind limb and intramuscular ganglioside injections were made into the same limb. ■, Gelatin-buffer i.v.; □, gelatin buffer i.m.; ▲, 5 mg. ganglioside i.v.; △, 5 mg. ganglioside i.m.

Fig. 6. A comparison of the effectiveness of ganglioside and ganglioside/cerebroside complex in protecting mice against tetanus resulting from the injection of vegetative bacilli of *Clostridium tetani* 5 hr before. The values are the means of the symptoms in each group of 20 mice (see Table 1). All the injections were intramuscular in the same hind limb. ●, Gelatin-buffer; △, 2 mg. ganglioside/cerebroside complex; □, 5 mg. ganglioside.

These experiments showed that ganglioside and ganglioside/cerebroside complex were effective prophylactic agents against the development of tetanus symptoms resulting from the injection of purified tetanus toxin 5 hr afterwards. If ganglioside were to be useful as a prophylactic agent in natural tetanus, it should be also be effective when introduced into the animal after the toxin. Figure 4 shows an experiment where a comparison was made of the protection afforded against 0·5 LD 50 of tetanus toxin by 5 mg. ganglioside or 2 mg. ganglioside/cerebroside (25 % ganglioside) complex injected either 5 hr before or 5 hr after the toxin. Both the ganglioside alone and the complex had more protective effect when injected before the toxin, but they still afforded protection when injected afterwards.

In considering natural tetanus it was also of interest to investigate whether ganglioside and ganglioside/cerebroside complex could protect mice against the development of symptoms resulting from the injection of live *Clostridium tetani* bacilli. The results

of such an experiment are shown in Fig. 5. Vegetative bacilli (equivalent to 1 LD 50 as far as could be determined) were injected, and 5 hr after this 5 mg. ganglioside was injected, either intramuscularly or intravenously. By either route this amount of ganglioside afforded some protection but, as in protection against the purified toxin, the intramuscular injection (same site of injection as the bacilli) was the more effective. Figure 6 shows the results of a comparable experiment where the protection by 2 mg. ganglioside/cerebroside complex, intramuscularly, was somewhat less than that by 5 mg. ganglioside (alone) by the same route.

DISCUSSION

The protective effect of ganglioside and of ganglioside/cerebroside complex against the effects of tetanus toxin injected intramuscularly was most marked when they were injected a few hours before, or at the same time, as the toxin, and at the same site. Also, 2 mg. of a complex containing 25 % ganglioside was as effective as 10 mg. of a complex containing 5 % ganglioside, and a complex containing 50 % ganglioside was ineffective. It would therefore appear that the protection afforded by these complexes reflects their ability to fix tetanus toxin at low concentrations of toxin (van Heyningen & Mellanby, 1968), although a complex containing 5 % ganglioside is relatively more effective in protecting mice against tetanus toxin than in fixing tetanus toxin *in vitro*. When the ganglioside/cerebroside complex was injected intravenously it did not protect mice from tetanus. The complex is not water-soluble and the mixture which was injected was a thick suspension. It is probable that the particles in this suspension would be too large to escape from the circulation into the tissues and could not reach the site where their action would be needed.

Since ganglioside and ganglioside/cerebroside complex were also effective in protecting against tetanus induced by the injection of vegetative bacilli of *Clostridium tetani*, they might have some prophylactic value in human tetanus. It must, however, be stressed that the protective agent would have to be injected as near the site of injury as possible, and as soon as possible (when free toxin was injected the protective effect was no longer found when treatment was delayed for 24 hr). Di- and tri-sialogangliosides containing two sialic acid residues attached to each other have a much greater affinity for tetanus toxin than the other major gangliosides of nervous tissue (van Heyningen, 1963). It is likely that their prophylactic value would also be greater, but at present it is difficult to separate them in amounts large enough for this to be tested.

We thank Dr R. O. Thomson of the Wellcome Research Laboratories for providing the toxin and for help with the growth of the organism and Mr P. A. Thompson for drawing the figures. This work was done during tenure of a contract (Task no. 136-474) between W. E. van Heyningen and the Office of Naval Research of the United States Department of the Navy.

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The Aerosol Survival and Cause of Death of *Escherichia coli* K12

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SUMMARY

The survival of *Escherichia coli* K12 HfrC sprayed from distilled water into a nitrogen atmosphere as a function of aerosol age and of storage relative humidity (RH) is demonstrated. The survival pattern was typically that of *E. coli*, i.e. marked instability in a region at high RH and better stability at low RH. The results of changing the RH from the storage RH to 100% or 30% are described. Comparison of survival in nitrogen with that in air showed air to be slightly toxic, the toxic component being oxygen or a trace of some contaminant in it. Glycerol and raffinose were slightly protective as additives at high RH; at low RH glycerol was toxic, but raffinose was highly protective. It was discovered that *E. coli* K12 HfrC carried a temperate phage and that this phage was not activated by the processes involved in aerosol experiments. The synthesis of phages T3 and $\mu 2$ by *E. coli* K12 HfrC, collected from the aerosol, was examined. Phage production and viability were similar, and hence it is concluded that in a nitrogen atmosphere loss of viability was not caused by DNA inactivation, DNA synthesis inhibition or inhibition of cell-wall division, but by failure of RNA synthesis, protein synthesis or energy production.

INTRODUCTION

Microbial survival in aerosols was reviewed in general terms by Anderson & Cox (1967). In particular, the aerosol survival of *Escherichia coli* has been found to depend upon several variables (Cox, 1965, 1966*a*, *b*, 1967, 1968; Cox & Baldwin, 1964, 1966, 1967). These include an 'air stress' (Cox 1966*a*) at low relative humidity (RH), caused by the toxic action of oxygen, possibly modified by the presence of contaminants (Cox & Baldwin, 1967; Hess, 1965; Webb, 1967). At high RH, regions were found where *E. coli* survival was particularly sensitive to RH, in a manner dependent on the spray fluid and collecting fluid (Cox, 1966*a*, *b*, 1967, 1968; Cox & Baldwin, 1966). Relative humidity changes imposed before collection of the aerosol also influenced survival, either beneficially or detrimentally, depending upon the strain of *E. coli* used and the nature of the spray and collecting fluids (Cox, 1966*b*, 1967). Such results showed that the death mechanisms of *E. coli* in nitrogen atmospheres must be influenced by the manner in which water re-enters the bacteria during collection, e.g. when *E. coli* B and COMMUNE (M.R.E. no. 162) are sprayed from raffinose into nitrogen at high RH (Cox, 1966*a*, *b*). Furthermore, it was shown that at high RH (where oxygen is not toxic, Cox & Baldwin, 1967) atmospheres of nitrogen, argon and helium were not completely biologically inert (Cox, 1968). Analysis of these results also indicated that the initial evaporation rates of the aerosol droplets did not influence the long-term survival of *E. coli* B in the aerosol. As a consequence it was thought likely that

RH was important with regard to survival through its effect on the water content of bacteria in the aerosol; RH and water content are related through the water sorption isotherm. Cox (1968) suggested that protective agents do not operate through a modification of the initial evaporation rate of aerosol droplets.

The present paper reports the aerosol survival as a function of RH of *Escherichia coli* K12 HfrC sprayed into air and into nitrogen. The object of the work was: first, to determine whether *E. coli* K12 HfrC behaved like other strains of *E. coli*, and, secondly, as it is a male-specific bacterium, to examine the ability of *E. coli* K12 HfrC collected from the aerosol to reproduce phage $\mu 2$ (RNA) and phage T3 (DNA). This approach has been previously reported for *E. coli* B and phage T7 (Cox, 1965; Cox & Baldwin, 1964, 1966; Webb, Dumasia & Singh Bhorjee, 1965).

METHODS

The techniques used were as previously reported (Cox, 1966*a*, *b*, 1967). *Bacillus subtilis* var. *niger* was used as a tracer (Anderson & Cox, 1967). At very high RH *B. subtilis* var. *niger* spores lose viability in the aerosol (Cox, 1966*a*). However, as discussed by Cox (1968), in order to obtain fine control of RH it is necessary to use a single-jet spray with, consequently, a low output. The only tracer method which is sufficiently sensitive is the spore tracer (Anderson & Cox, 1967). Aerosol samples for assay of phage $\mu 2$ and phage T3 synthesis were collected in liver-digest broth + anti-foam in place of the more usual phosphate buffer or phosphate buffer + sucrose (Cox, 1966*a*). Samples (2 ml.) of aerosol were warmed to 37°, and suitably diluted. Phage $\mu 2$ and phage T3 were added to respective samples to give a phage: bacterium ration of 3 : 1. As a control, the spray fluid was diluted to give the same number of coli organisms/ml. as in the aerosol samples, and was then treated similarly to the aerosol samples. Following incubation for 2 hr. at 37° serial dilutions were made and 1 ml. was added to 2 ml. blood agar base (0·7% agar, tryptone, liver digest, yeast extract, sodium chloride) at 45°. The diluted agar was seeded with *Escherichia coli* K12 HfrC and poured on Petri dishes containing blood agar base (1·5% agar). After incubation for 18 hr. at 37° the plaques were counted. Six plates/dilution were used.

Previous studies (Cox & Baldwin, 1964, 1966) of the ability of *Escherichia coli* B to reproduce phage T7 were made by direct observation of *E. coli* B with a microscope. This technique was found to be unsuitable for *E. coli* K12 HfrC because lysis was not as definite as with *E. coli* B.

RESULTS

Escherichia coli K12 HfrC sprayed from distilled water

Figures 1 to 3 give the survival in nitrogen at 0·3 sec., 2 min., 15 min. and 30 min. collected into phosphate buffer. Figure 3 also gives the result for a collecting fluid of phosphate buffer + M-sucrose and indicates a similar phenomenon to *Escherichia coli* JEPP, namely, that at low RH the addition of sucrose to the collecting fluid decreased survival. The survival pattern was typically that for *E. coli*, namely, a region at high RH where *E. coli* was particularly unstable, while at low RH greater survival was obtained. The unstable region at high RH was particularly marked at an aerosol age of 2 min., where at 85% RH the survival was 0·13%. As the aerosol aged the minimum widened to give 0% survival at RH 85–70% inclusive. Since the minimum was less

critical with regard to RH than was found with *E. coli* B, JEPP and COMMUNE (M.R.E. no. 162), this K12 strain of *E. coli* was ideally suited to a study of the mechanism which caused such a minimum.

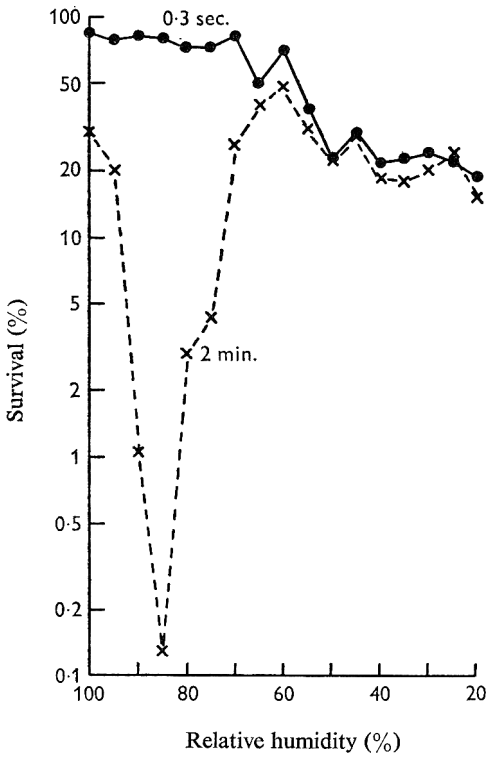

Fig. 1

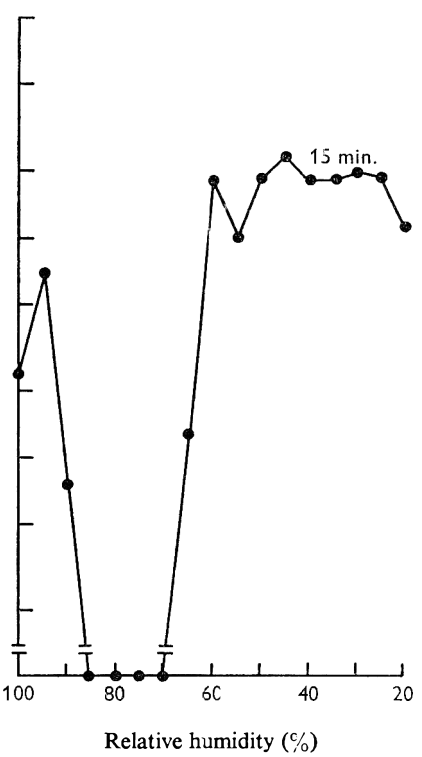

Fig. 2

Fig. 1. The aerosol survival of *Escherichia coli* K12 HfrC sprayed from distilled water into nitrogen at 26·5° as a function of relative humidity. ●, 0·3 sec. aerosol age; ×, 2 min. aerosol age. Phosphate buffer collecting fluid.

Fig. 2. The aerosol survival of *Escherichia coli* K12 HfrC sprayed from distilled water into nitrogen at 26·5° as a function of relative humidity, at an aerosol age of 15 min. Phosphate buffer collecting fluid.

Figure 4 gives the results for RH changes from the storage RH to either 100% or 30% prior to collection of the aerosol. A change to 100% RH caused little improvement in survival at high RH and lowered survival at low RH. Conversely, a change to 30% RH lowered survival at high RH and at low RH had little effect. These results indicate that the rehydration process during collection can be very important with regard to survival.

Table 1 compares the survival at 30 min. aerosol age for *Escherichia coli* K12 HfrC sprayed from distilled water into air and into nitrogen. It is evident that air was slightly toxic, and by using mixtures of nitrogen and oxygen the lower survival in air was shown to be caused by oxygen, or a trace contaminant in it (see also Cox & Baldwin, 1967).

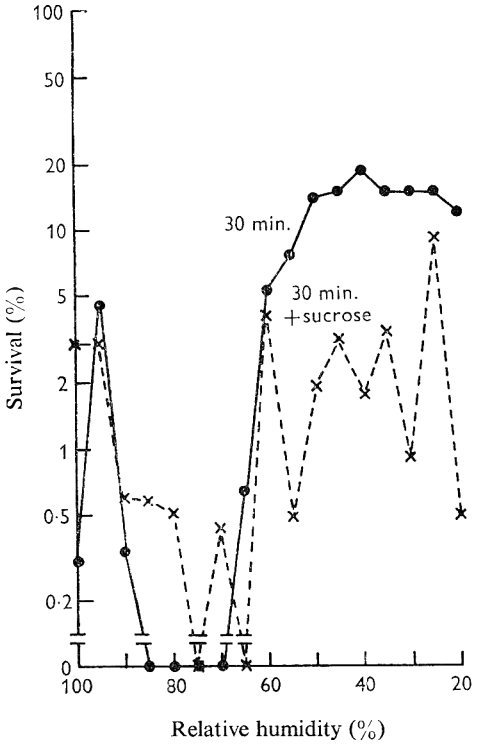

Fig. 3

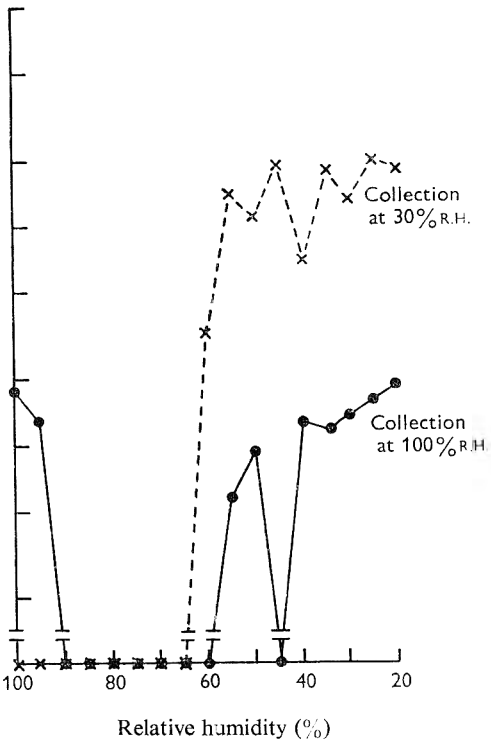

Fig. 4

Fig. 3. The aerosol survival of *Escherichia coli* K12 HfrC sprayed from distilled water into nitrogen at 26·5° as a function of relative humidity, at an aerosol age of 30 min. ●, Phosphate buffer collecting fluid; ×, phosphate buffer + M-sucrose collecting fluid.

Fig. 4. The aerosol survival of *Escherichia coli* K12 HfrC sprayed from distilled water into nitrogen at 26·5° as a function of relative humidity, at an aerosol age of 31·5 min. ●, Relative humidity changed to 100% before collection; ×, relative humidity changed to 30% before collection. Phosphate buffer collecting fluid.

Table 1. *The survival of Escherichia coli K12 HfrC sprayed from distilled water at an aerosol age of 30 min., as a function of relative humidity*

Collecting fluid was phosphate buffer. Temperature 26·5°.

Relative humidity (%)	95	85	75	65	55	45	35	25	20
Survival in air (%)	1·2	0	0	0	4·4	7·0	10	3·0	7·2
Survival in nitrogen (%)	4·5	0	0	0·64	7·8	15	16	16	12

Escherichia coli K12 sprayed with protective agents

Previous studies (e.g. Cox, 1967) investigated the protective action of glycerol and of raffinose. These substances were chosen because glycerol readily permeated *Escherichia coli*, whereas raffinose only very slowly penetrated the cell wall. Their effects on *E. coli* K12 HfrC are given in Table 2, together with the survival for *E. coli* K12 HfrC sprayed from distilled water. The data show that glycerol was slighly protective at high RH and toxic at low RH; raffinose showed slight protection at high RH and good

protection at low RH. This pattern has been found with other strains of *E. coli* (e.g. Cox 1967).

Table 2. *The survival of Escherichia coli K12 HfrC sprayed from protective agents as a function of aerosol age and of storage relative humidity*

Collecting fluid was phosphate buffer, except: (*) which was phosphate buffer + M-sucrose; (†) RH changed to 100% prior to collection; (‡) RH changed to 30% prior to collection. Glycerol was equilibrated with the bacteria for 15 min. before spraying. Raffinose was added immediately before spraying. Temperature 26·5°.

	% survival											
	Glycerol, 0·3 M				Raffinose, 0·15 M				Distilled water			
Spray fluid RH (%)	85	75	45	25	85	75	45	25	85	75	45	25
Aerosol age												
0·3 sec.	98	63	26	38	93	86	85	85	80	73	38	22
2 min.	15	3·2	0	0·9	15	48	81	92	0·13	4·5	31	26
15 min.	6·4	1·3	0	4·4	0	16	75	79	0	0	9·4	19
30 min.	3·6	0	0	3·1	1·0	4·8	69	84	0	0	7·9	17
30 min.*	17	2·2	0	6·6	4·9	5·5	34	28	0·58	0	0·41	8·4
31·5 min.†	17	0	0	0	0	3·4	39	38	0	0	0·58	1·6
31·5 min.‡	1·7	0	0	0	0	3·4	73	79	0	0	14	20

Table 3. *The synthesis of phage T3 and phage μ2 by Escherichia coli K12 HfrC collected from the aerosol*

Aerosol age 30 min.; temperature of storage 26·5°. Collecting fluid liver-digest broth + anti-foam.

RH of aerosol (%)	Phage T3 production (%)	Phage μ2 production (%)	Viability (%)
85	0	0	0·5
30	22	9	14

The temperate phage of Escherichia coli K12 HfrC

It was suspected that *Escherichia coli* K12 HfrC might carry a temperate phage. Ultraviolet irradiation or heating at 50° for 30 min. activated the temperate phage, which readily plaqued on *E. coli* K12 HfrC and *E. coli* B, suggesting that it was a DNA phage. This situation gave rise to another possible death mechanism for *E. coli* K12 HfrC, namely that aerosolization activated the temperate phage, which then directed the metabolism of *E. coli* K12 HfrC and prevented colony formation. But, although activation of the temperate phage was looked for at high, intermediate and low RH, none was found. Hence this death mechanism did not occur for *E. coli* K12 HfrC, but may occur with other bacteria and should not be overlooked.

The synthesis of phage T3 and phage μ2 by Escherichia coli K12 HfrC collected from the aerosol

Table 3 gives the % phage production and % viability. The data show that at 85% RH neither phage T3 nor phage μ2 was produced, while at 30% RH phage production was similar to the extent of colony formation. Therefore, death of *Escherichia coli* K12 HfrC can be attributed to the failure of a metabolic function which was common to the multiplication of *E. coli* K12 HfrC and to the production by this bacterium of phages T3 and μ2.

DISCUSSION

In general, the survival of *Escherichia coli* K12 HfrC followed the pattern for *E. coli* B JEPP and COMMUNE (M.R.E. no. 162). Of the four coli strains studied, *E. coli* K12 HfrC was the most unstable at high and at low RH, and was a good organism with which to study death mechanisms other than oxygen toxicity (Cox & Baldwin, 1967), because of the extent of viability loss when sprayed from distilled water into a nitrogen atmosphere. The minimum at 85% RH was very similar to that found at the same RH for *E. coli* B, but was very apparent at a much shorter aerosol age. The effect of the collecting fluid was also similar to that for *E. coli* JEPP (Cox, 1966*a*, *b*, 1967). This effect, and that caused by RH changes before collection, indicated, as for the other three strains of *E. coli* studied, that the manner in which water enters the bacterium during collection influences the survival. Oxygen toxicity was less apparent with *E. coli* K12 HfrC than, for example, *E. coli* B (Cox, 1966*a*; Cox & Baldwin, 1967), probably owing to the poor survival of the former in nitrogen at low RH (in the absence of protective agents). The action of glycerol and raffinose was very similar to that with *E. coli* JEPP (Cox, 1967), and again showed that at low RH a protective agent (namely raffinose) outside the cell wall was able to confer stability.

The fact that *Escherichia coli* K12 HfrC carried a temperate phage suggested an interesting death mechanism, although in this case it was shown to be inoperative. Hayes (1964) reported that *E. coli* B carried a prophage, but its activation as a cause of death of *E. coli* B can probably be excluded since Cox & Baldwin (1966), who observed *E. coli* B collected from the aerosol onto a nutrient agar by means of a microscope, found that *E. coli* B did not lyse. However, it is possible that the *E. coli* B phage was activated and reproduced without lysis of the *E. coli* B. The results obtained for phage T3 and phage $\mu 2$ production by *E. coli* K12 HfrC collected from the aerosol strongly suggest that neither DNA inactivation, DNA synthesis inhibition nor inhibition of cell-wall division were the cause of the loss of viability of *E. coli* K12 HfrC in nitrogen. Failure of these processes would not prevent phage production, especially as phage $\mu 2$ is an RNA phage which can replicate independently of DNA metabolism. It is therefore possible to conclude that the cause of death of *E. coli* K12 HfrC as an aerosol in nitrogen was because of failure of RNA synthesis, protein synthesis or energy production. The finding that DNA metabolism was not involved in the death mechanism is in agreement with the results of Benbough (1967), who used an entirely different technique. Since DNA inactivation is unlikely, then the results of Cox & Baldwin (1966) can be interpreted to suggest strongly that the toxic action of oxygen is to interfere with cell-wall synthesis and cell division.

The author thanks Mr C. M. Saunders for technical assistance.

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The Detection of a Strain of *Chromobacterium lividum* in the Tissues of Certain Leaf-nodulated Plants by the Immunofluorescence Technique

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SUMMARY

An organism was isolated in pure culture from germinating seeds of *Psychotria nairobiensis* Brem. By standard bacteriological techniques the organism was considered to belong to the *Chromobacterium lividum* group. It was shown to fix nitrogen. By using an antiserum prepared against one strain, four strains were found to be serologically identical and showed some cross-reaction with *Agrobacterium tumefaciens*. An immunofluorescence technique was used in a search for these organisms in the seed embryo, leaf nodules and other tissues of a number of leaf-nodulated Rubiaceae and Myrsinaceae including *Psychotria nairobiensis*, *Ardisia crispa* and a non-nodulated species of Myrsinaceae, *Ardisia elliptica*. From the results obtained it is concluded that this serotype of *Chromobacterium lividum* is present in the leaf-nodulated members of these families of plants.

INTRODUCTION

Although investigations of the organisms in leaf-nodule symbiosis of tropical Rubiaceae and Myrsinaceae species have been made for the last eighty years (von Höhnel, 1882; Solereder, 1899; Trimen, 1894; Miehe, 1911*a*, *b*, 1914, 1919; von Faber, 1912, 1914; Boodle, 1923; de Jongh, 1938; Hanada, 1954; Bose, 1955; Silver & Stewart, 1962; Centifanto & Silver, 1964) the evidence put forward by these authors leaves the identity and significance of the bacterium still undecided. It is claimed that closely similar conditions have been produced in many plant species by bacteria of different genera but in which each genus is related to a particular host.

The bacterial genus *Rhizobium* is known to be associated with root-nodule formation in many members of the plant family Leguminosae. In this family, plants free from bacteria may be readily obtained and successful re-infection may therefore be done. So far plants of the families Rubiaceae and Myrsinaceae have not been convincingly shown to yield plants free from bacteria; and, in each leaf-nodulated species investigated, infection has been shown to be systemic. The seeds contain bacteria which are not removed by surface sterilization. Gordon (1963) showed that seeds of *Psychotria nairobiensis* (Rubiaceae) heated at 50° for 10 min. and then grown under normal

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tropical greenhouse conditions produced not only normal leaf-nodulated plants but also crippled dwarf plants, apparently without leaf nodules. These crippled plants eventually, after 9 months observation, either reverted to normal leaf-nodulated plants or died without leaf-nodule formation. Earlier workers (von Faber, 1912, 1914; Miehe, 1911*a*, *b*, 1914, 1919; de Jongh, 1938; Silver & Stewart, 1962; Centifanto & Silver, 1964) used crippled plants produced in a similar fashion for re-infection experiments, but no precautions were taken against adventitious infections, nor was it appreciated that such plants might revert to normal.

As both cultural and re-infection experiments had given inconclusive results, it was decided to use the fluorescent antibody technique for the identification and study of the bacteria in leaf nodules and other tissues of Rubiaceae and Myrsinaceae.

During the study of bacteria associated with the tissues of *Psychotria nairobiensis* (Rubiaceae) and *Ardisia crispa* (Myrsinaceae) (Gordon, 1963) bacteria provisionally placed in the family Rhizobiaceae were consistently isolated from germinating seeds. It was impossible to isolate these organisms from the leaf nodules, although they were observed there by staining techniques. The present work describes the fluorescent antibody technique to detect organisms in the various tissues of these plants and other members of the Rubiaceae and Myrsinaceae.

METHODS

Plant materials. Specimens of *Ardisia crispa* strain A.DC., *Psychotria emetica* and *Pavetta grandiflora* were obtained from the Chelsea Physic Garden by kind permission of Mr W. McKenzie. *Psychotria nairobiensis* plants were grown from seed kindly given by Dr B. Verdcourt (East African Agricultural and Forestry Research Organization, Nairobi, Kenya). *P. hirtella*, *P. kirkii*, *P. capensis*, *Pavetta gardeniifolia*, *Pav. termitaria*, *Pav. revoluta*, *Pav. lanceolata* and the non-nodulated *A. elliptica* were obtained from the Royal Botanic Gardens, Kew, by kind permission of the Director.

Reference bacterial cultures. The following cultures were tested as they were known to be symbionts or associated with plant diseases, or were possibly taxonomically related to our isolates. Cultures of *Rhizobium leguminosarum* strain 317, *R. meliloti* strain AH2, *R. lupini* strain LYNGBY, *R. phaseoli* strain CN, *R. trifolii* strain CLF and *R. japonicum* strains B54 and CMR15 were obtained from the Rothamsted Experimental Station, Harpenden, Hertfordshire, by kind permission of Dr P. S. Nutman. *Enterobacter aerogenes*, *Azotobacter vinelandii*, *Pseudomonas fluorescens*, *Agrobacterium tumefaciens* strains 4 and 8, *Xanthomonas begoniae* and *Proteus vulgaris*, were obtained from the culture collection of the Bacteriology Department, Imperial College, London. *Escherichia coli* strain E65/56 (026.B6.H-) and *Salmonella typhimurium* strain SAL. 1223/67 were obtained from the Salmonella Reference Laboratory, Colindale. *Klebsiella rubiacearum* was obtained from the University of Florida through Dr W. S. Silver. *Klebsiella* sp. serotype 24 (NCTC 9144) was obtained from the National Collection of Type Cultures, Colindale, through the late Dr K. J. Steel, and *Chromobacterium violaceum* (NCTC 9757), *C. lividum* (NCTC 9796), *C. typhiflavum* (NCTC 9381) and *Flavobacterium meningosepticum* (NCTC 10016) through Dr S. P. Lapage.

Isolation of bacterial cultures from plants. Seeds of *Ardisia crispa* and *Psychotria nairobiensis* were permitted to germinate in a moist chamber at 25° for 20 days and then sterilized by 0·1% mercuric chloride, washed 10 times with sterile distilled

water and each seed was crushed in 2 ml. sterile distilled water (Gordon, 1963). These suspensions were plated on to Oxoid nutrient agar and incubated at 25°. After 2 days a practically pure culture of mucoid colonies often with violet pigmentation, which rapidly changed to pale yellow on subculture, was observed (Gordon, 1963). Gram-negative organisms morphologically similar to those in the colonies were seen in living plants and their seeds by microscopic examination of variously stained sections (Gordon, 1963).

The biochemical reactions of two bacterial colonies from each species of seed were investigated by using the methods of Cowan & Steel (1965). The ability to grow on synthetic nitrogen-free medium (Gordon, 1963) was also investigated. In addition, nitrogen fixation studies were performed by Dr E. R. Roberts using ^{15}N and a mass spectrometer, and it was found that our isolates fixed nitrogen (Gordon, 1963).

As discussed later these isolates were classified as *Chromobacterium lividum*; they will be referred to as 'our isolates'.

Preparation of antigens. The strain of Chromobacterium isolated from *Psychotria nairobiensis* was grown as a lawn on Oxoid nutrient agar plates incubated at 25° for 24 hr followed by harvesting of the bacteria in physiological saline and washing three times with saline. The bacteria were then suspended in saline and immediately prior to the addition of 0·5% (v/v) formalin a colony count was done, showing that 4×10^8 bacteria/ml. were present.

Preparation of the antiserum. Before any inoculations blood was withdrawn from the marginal vein of the rabbit ear to give a sample of normal serum. The inoculation course consisted of increasing doses of antigen (0·5, 1·0, 2·0, 4·0 ml.) given at intervals of 5 days. These inoculations were into the marginal vein of the ear. Three days after the last injection blood was withdrawn again from the rabbit; the agglutinin titre of the serum prepared from it was rather low, therefore this antiserum was not used. A second and similar course of inoculations was given to the same rabbit, after which a higher agglutinin titre, 1/160, was obtained. After the second course, two withdrawals of blood were made at 3-day intervals and the rabbit was then bled out. The serum obtained from these samples of blood was pooled and used as the source of specific antibody for all subsequent work.

Gel-diffusion studies. Soluble antigen preparations of organisms were prepared by scraping growth from a 2-day culture on nutrient agar and shaking in toluene for 1 hr at 37°. The aqueous layer contained the antigen material used in these studies.

The tests were done on microscope slides each covered with 5 ml. agar of the following composition: 0·02 M-sodium phosphate buffer (pH 6·0), 0·01% (w/v) sodium azide and 1·2% (w/v) Oxoid Ion agar no. 2. The slides were placed in a moist chamber at room temperature and examined daily for a week for the production of precipitin lines.

Preparation of the fluorescein isothiocyanate (FITC)-labelled specific antibody. The normal serum and the antiserum were conjugated with FITC by the method described by Taylor, Heimer, Lea & Tomlinson (1964). Thus, from the same rabbit pre-inoculation and hyperimmune globulins were conjugated with FITC. Some conjugated hyperimmune serum was kept for the blocking test.

Preparation of materials for microscopic examinations. The plant tissues were immersed in 95% (v/v) ethanol in water for 1 min. followed by washing with agitation in sterile distilled water for 5 min. Films of macerated plant tissues or films of pure cultures of bacteria were made on grease-free thin slides, which were dried at room

temperature and then fixed in methanol for 10 min. After the methanol had evaporated, one drop of FITC-labelled γ-globulin was applied to each film. The slides were then incubated in a moist chamber at 37° for 20 min., washed in running tap water and blotted dry.

Microscopic examinations. Most of the preparations were examined in a darkened room with a Watson Bactil microscope fitted with a dark-ground condenser, Carl Zeiss fluorescence-free immersion oil being used with this microscope, which was adapted for fluorescence microscopy as described by Taylor & Heimer (1964). A 805Q Ilford colourless gelatin ultraviolet filter was used. The light was a 250W. ME/D mercury vapour compact source lamp with an OX 1 Chance Pilkington glass excitation filter attached.

Other preparations were examined under ultraviolet radiation with a Reichert Zetopan Universal Fluorescence Microscope fitted with a dark-ground condenser, BG 12/4 mm. excitation filter, and an orange barrier filter. A $\times 8$ eyepiece and a $\times 100$ objective (numerical aperture 1·18) were used. Fluorescence-free liquid paraffin was placed between the dark-ground condenser and the slide, and Analar glycerol between the slide and objective. Other microscopic observations were made with a Gillet and Sibert Conference Microscope, with an Iodine Quartz lamp with Kohler illumination, a cardioid condenser, a 2 mm. BG 12 primary filter and a GG 5 secondary filter of Schott glass. A $\times 5$ photo eyepiece and a $\times 100$ achromat objective (numerical aperture 1·3) and an iris diaphragm were used.

RESULTS

Bacteriology

The morphological, cultural and biochemical characters of two isolates from germinating seeds of *Psychotria nairobiensis* and two isolates from *Ardisia crispa* were identical (Gordon, 1963). All four strains had been shown to fix nitrogen by Dr E. R. Roberts.

The biochemical reactions were investigated of the following bacterial cultures: *Rhizobium leguminosarum*, *R. meliloti*, *R. lupini*, *R. phaseoli*, *R. trifolii*, *R. japonicum*, *Chromobacterium violaceum*, *C. typhiflavum*. *Enterobacter aerogenes*, *Azotobacter vinelandii*, *Pseudomonas fluorescens*, *Agrobacterium tumefaciens*, *Xanthomonas begoniae*, *Proteus vulgaris*, *Escherichia coli*, *Salmonella typhimurium*, *Klebsiella rubiacearum*, *Klebsiella* sp. serotype 24, and *Flavobacterium meningosepticum*. The reactions differed on numerous tests from our isolates, which most closely resembled *C. lividum*.

Our isolates gave the following results: Gram-negative rods 1·5–3·5 × 0·3–0·5 μ, motile, capsulated. Produce violet pigment on first isolation; yellow pigment later; oxidase and catalase positive; growth range 4–30°, therefore psychrophilic; do not attack the following carbohydrates: fructose, glucose, trehalose, lactose, mannitol, sucrose, salicin, dulcitol, inositol, adonitol, raffinose, sorbitol, arabinose, rhamnose, xylose, inulin, glycerol, cellobiose, sorbose, starch, maltose, glycogen and galactose. Hugh and Leifson test negative. Reduce nitrate to nitrite. Utilize citrate as sole carbon source and fix nitrogen. Grow on nutrient agar. Do not grow on MacConkey agar. Do not produce indole, H_2S or urease; give negative methyl-red, Voges–Proskauer, and KCN tests; do not utilize malonate, liquefy gelatin, oxidize gluconate, deaminate phenylalanine, or decarboxylate arginine, lysine or ornithine; give a negative o-nitrophenyl-β-galactoside test.

Gel-diffusion studies

Soluble antigen preparations of our four isolates were tested by gel-diffusion against antiserum. Each of the four strains produced a single precipitin line and the four lines joined, proving that all four strains were identical.

Soluble antigen preparations were also made from the bacteria listed in Table 2; all results were negative except that a faint precipitin line was produced by *Agrobacterium tumefaciens* which suggested an antigenic relationship between this species and our isolates.

FITC-staining results

In microscopic examinations the following criteria were used: when bacteria were strongly fluorescent and apple green in colour, specifically labelled bacteria were suspected of being present; non-specific staining was recorded for those bacteria which were non-fluorescent and dull green to grey-green in colour. These results are shown in Table 1.

Table 1. *Results of examination of bacterial cultures by means of fluorescent antibody staining and gel-diffusion*

Organism	Specific fluorescence	Precipitin line
Chromobacterium lividum isolated from:		
Psychotria nairobiensis A	+	+
P. nairobiensis B	+	+
Ardisia crispa A	+	+
A. crispa B	+	+
Agrobacterium tumefaciens 4	±	±
A. tumefaciens 8	±	±

The following gave a negative reaction in both tests: *C. lividum* (NCTC 9796), *C. violaceum*, *Rhizobium lupini*, *R. meliloti*, *R. leguminosarum*, *R. phaseoli*, *R. trifolii*, *R. japonicum*, *Enterobacter aerogenes*, *Azotobacter vinelandii*, *Pseudomonas fluorescens*, *Xanthomonas begoniae*, *Proteus vulgaris*, *Escherichia coli*, *Salmonella typhimurium*, *Klebsiella rubiacearum*, *Klebsiella* sp. serotype 24, *Flavobacterium meningo-septicum*, *C. typhiflavum*.

Two control tests were carried out on slides of the same material as that used to determine whether the fluorescent staining reaction was specific. γ-globulin was prepared from the non-immune serum, conjugated with FITC and applied to films of plant tissue; no specifically stained bacteria were observed, but some autofluorescence was seen which was identical to that obtained by using labelled specific γ-globulin.

That the labelled antibody was specific in its staining reaction of our isolates was proved by the 'blocking test' (Mellors, 1959). Plant tissue preparations similar to those used above were treated with unconjugated specific γ-globulin, then stained with labelled γ-globulin. In every case the unconjugated specific γ-globulin blocked staining by the labelled specific γ-globulin. Further tests showed that the hyperimmune γ-globulin failed to react with a wide selection of Gram-negative rod-shaped bacteria (Table 1). These tests confirmed that the hyperimmune γ-globulin was specific for our isolates. Of the other organisms examined (Table 1) only *Agrobacterium tumefaciens* reacted. These organisms were slightly stained by hyperimmune FITC-conjugated γ-globulin, suggesting an antigenic relationship with our isolates. This view was supported by the results of the gel-diffusion tests also given in Table 1.

Plant tissues. The following plant tissues were examined: crushed seed embryos, crushed leaf nodules, crushed flowers (Table 2). Films of these macerated tissues from nodulated and non-nodulated plants were treated with hyperimmune FITC conjugated γ-globulin; the results are shown in Table 2. In the leaf-nodulated plants 95–100% of the organisms present were stained specifically with FITC-labelled specific antiserum, showing that serotypes identical with our isolates, which we have identified as *Chromobacterium lividum*, were present in the nodules and all other tissues examined of the nodulated species listed in Table 2. This serotype was not seen in the non-nodulated Myrcinaceae species, *Ardisia elliptica.*

Table 2. *Results of fluorescent staining of plant tissues*

Plant tissues examined	Specifically fluorescing organisms present and constituting 95–100% of all visible organisms
Psychotria nairobiensis	
Crushed seed embryo	+
Crushed leaf nodules	+
P. emetica	
Crushed seed embryo	+
Crushed leaf nodules	+
P. hirtella	
Crushed seed embryo	+
Crushed leaf nodules	+
Crushed flowers	+
P. kirkii	
Crushed leaf nodules	+
P. capensis	
Crushed leaf nodules	+
Pavetta gardenifolia	
Crushed leaf nodules	+
Pav. grandiflora	
Crushed leaf nodules	+
Crushed flowers	+
Pav. revoluta	
Crushed leaf nodules	+
Pav. lanceolata	
Crushed leaf nodules	+
Ardisia crispa	
Crushed seed embryo	+
Crushed leaf nodules	+
**A. elliptica*	
Crushed leaves	−

* non-nodulated species

In most of the macerated plant tissues which contained specifically staining bacteria a considerable quantity of apple-green fluorescent slime was also observed. This slime was not seen in plant tissues stained according to the 'blocking test' or with labelled non-immune antiserum. It is therefore considered that this slime contains specific antigenic material produced by or originating from these bacteria. Our isolates also formed a slime in artificial media under certain conditions (Gordon, 1963).

DISCUSSION

On the basis of the work described it is considered that the fluorescent antibody tracing technique is valuable in cases such as the present where it is difficult if not impossible to apply Koch's postulates. During the past 80 years, many different bacteria have been isolated from leaf-nodulated plants but re-infection experiments were done under conditions which allowed spontaneous reversion to nodulation to occur. Therefore it is considered that these results are open to doubt.

It has been shown that a single serotype of bacteria classified in the *Chromobacterium lividum* group was present in all tissues examined of the leaf-nodulated plants listed in Table 2 and that these bacteria were the bacteria observed. Because of these results it is considered that this serotype was the symbiont of the nodulated plants listed (Table 2). Further evidence was provided by the fact that the leaves of the non-nodulated member of the family Myrsinaceae *Ardisia crispa* did not contain any bacteria which stained with this FITC-labelled specific antibody, neither were other organisms detected using standard methods.

The biochemical reactions of our isolates show that they belong to the family Rhizobiaceae as described by Moffett & Colwell (1968). The difficulty of distinguishing clearly between the families Rhizobiaceae and Pseudomonadaceae is discussed in their paper and they point out that the root-nodule bacteria might better be placed in the family Pseudomonadaceae. They also suggest that *Chromobacterium lividum* might be a separate genus. Sneath (1956) gives his results in a detailed study of the *Chromobacterium* group, which he divides into the mesophilic group, *C. violaceum*, and the psychrophilic group, *C. lividum*. Our isolates gave reactions identical with the latter. In the Hugh & Leifson test prepared according to the method given in Cowan & Steel (1965) our strains gave a negative result at 25° but an oxidative reaction at 37°. These results were confirmed in the National Collection of Type Cultures, where ten strains of *Chromobacterium lividum* were also examined and it was found that two strains gave a negative reaction in Hugh & Leifson at 25° and at 37°.

Sneath (1956) had found that these two strains, CA and EB, gave oxidative reactions in Hugh & Leifson's medium at 25° but this is possibly due to slight differences in the ingredients of the medium.

The extensive cultural and morphological characteristics given by Gordon (1963) also agree closely with the description given by Sneath (1956) for the *Chromobacterium lividum* group. Bacteria of the Rhizobiaceae are often difficult to classify, particularly if found unassociated with their normal plant host (Skerman, 1967), therefore the fact that our isolates are similar to *C. lividum* makes us conclude that they belong to that group.

One of the strains isolated from *Psychotria nairobiensis* has been deposited in the National Collection of Type Cultures, number NCTC 10590, and one of the strains isolated from *Ardisia crispa*, number NCTC 10591.

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Aflatoxin B_1 Binding and Toxic Effects on *Bacillus megaterium*

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SUMMARY

A study was made of the binding of aflatoxin B_1 and its toxic effects on *Bacillus megaterium* NRRL B-1368. After a 12 hr incubation period, 5 μg. aflatoxin B_1/ml. inhibited growth of the test organism 95%. Viable organisms decreased from $3{\cdot}8 \times 10^7$/ml. in the controls to $5{\cdot}0 \times 10^4$/ml. in a growth medium containing 50 μg. aflatoxin B_1/ml., after 4 hr. of incubation. Viability was not significantly decreased, however, when the organisms were incubated with the same concentration of toxin either under nitrogen, or at 5°, or in the presence of bacteriostatic concentrations of tetracycline. Binding experiments showed that $4{\cdot}0 \times 10^9$ organisms/ml. took up approximately 7·0 μg. aflatoxin B_1/ml. After five aqueous washes of the organisms, 20–25% of the toxin remained bound to them. The tightly bound toxin was removed by ultrasonic treatment of the organisms and chloroform extraction of the macerate. Removal of cell walls by lysozyme following toxin uptake released 23% of the initially bound toxin, and osmotic rupture of the protoplasts an additional 7%. Toxin was also taken up by intact and by ruptured protoplasts. Removal of nucleic acids from the membranes did not alter their capacity to bind aflatoxin B_1.

INTRODUCTION

The uptake of aflatoxin B_1 by *Flavobacterium auranticum* NRRL B-184 and the resulting toxic effects have been investigated (Ciegler, Lillehoj, Peterson & Hall, 1966; Lillehoj, Ciegler & Hall, 1967). These studies showed that 10^{11} intact organisms/ml. removed 7·0 μg. aflatoxin B_1/ml. from an aqueous medium. Autoclaved organisms and cell-wall preparations also bound the toxin, which could be recovered from them by washing; toxin taken up by intact organisms could not be entirely extracted. It was concluded that aflatoxin B_1 is initially bound loosely to the cell wall, followed by a tighter binding to sites in the intact organisms.

Aflatoxin B_1 has shown a facility for binding to deoxyribonucleic acid (DNA) (Clifford & Rees, 1966; Sporn, Dingman, Phelps & Wogan, 1966; Black & Jirgensons, 1967). These studies and others have related the cytotoxic aspects of aflatoxin to a DNA binding followed by a perturbation of messenger ribonucleic acid (RNA) synthesis. Binding of aflatoxin B_1 to DNA, however, is not exclusive since it has been observed that it also binds to RNA (Sporn *et al.* 1966) and to histones (Black & Jirgensons, 1967). The nature of the binding of aflatoxin B_1 to DNA has been examined by several investigators. Some workers believe that aflatoxin B_1 functions as an alkylating agent (Legator, Zuffante & Harp, 1965; Wragg, Ross & Legator, 1967). But studies on the stability of the DNA–aflatoxin B_1 complex have shown that the toxin can be separated from the nucleic acid on a Sephadex-50 column (Clifford & Rees, 1967). There is also no effect on the melting profile of the DNA produced by the interaction with the aflatoxin.

The purpose of the present work was to acquire further information about the nature of uptake and binding of aflatoxin B_1 and its toxic effects on a sensitive microorganism. Since determination of toxic uptake by protoplasts and protoplast membranes seemed desirable, *Bacillus megaterium* NRRL 1368 was chosen as the test organism; it is sensitive to aflatoxin B_1 (Burmeister & Hesseltine, 1966) and readily yields protoplasts by the action of lysozyme.

METHODS

Production and harvest of bacilli. Inhibition studies of *Bacillus megaterium* were done in the malt-extract + yeast-extract medium (MY) described by Haynes, Wickerham & Hesseltine (1955). Ten ml. medium in 300 ml. Erlenmeyer flasks were inoculated with $1{\cdot}0 \times 10^8$ bacilli followed by incubation at 30° on a rotary shaker. Growth was measured by determining the extinction (540 mμ, Beckman B spectrophotometer) of a fixed volume of culture medium. Protoplasts were produced from bacilli grown in a medium containing (w/v): peptone (Difco Laboratories, 920 Henry Street, Detroit, Mich.) 2·0 %, sodium chloride 0·5 % (when necessary solidified with 2 % agar). Five hundred ml. of this medium in 2·8 l. Fernbach flasks were inoculated with $1{\cdot}5 \times 10^{10}$ bacilli followed by incubation at 30° for 4 hr on a rotary shaker. Bacilli for protoplast formation were harvested by centrifugation at 5000 *g* for 1 hr.

Counting of viable organisms. Ten ml. MY growth medium in 300 ml. Erlenmeyer flasks were inoculated with stationary-phase bacilli and incubated on a rotary shaker at 30°. Successive tenfold dilutions in growth medium were made of samples of culture. Three samples were taken from an appropriate dilution and spread on growth-medium agar in Petri plates which were incubated for 14 hr at 28°. Colony counts were only recorded on plates which showed 10 to 100 colonies.

Formation and lysis of protoplasts. Eighty-two ml. of 2 M-sucrose, 4 ml. of M-sodium chloride and 100 mg. lysozyme (Calbiochem, Box 54282, Los Angeles, California) were added to 250 ml. phosphate buffer (pH 7·0) containing $2{\cdot}0 \times 10^{11}$ *Bacillus megaterium* organisms (Weibull & Bergström, 1958). Protoplast formation was followed microscopically and was generally complete in 1 hr. The protoplasts were centrifuged down (15,000 *g* for 30 min.) and then lysed by suspension in 0·05 M-tris (pH 7·6) + 0·005 M-magnesium chloride, followed by homogenization with a tissue-grinder. Protoplasts were washed in isotonic medium before testing, and membranes were routinely washed twice with tris buffer (pH 7·6) before aflatoxin uptake studies.

Aflatoxin B_1 production, assay and binding procedures. Aflatoxin B_1 was produced and recovered according to the procedures of Shotwell, Hesseltine, Stubblefield & Sorenson (1966). A standard solution of aflatoxin B_1 was prepared in ethanol and diluted as required in the desired medium. In growth studies the ethanol was removed from the medium under vacuum, before inoculation. Only concentrations of aflatoxin B_1 which exhibited a coefficient near that reported at 363 mμ (eM = 22,000) were used (Nabney & Nesbitt, 1965). Toxin concentrations were determined by thin-layer chromatography (Shotwell *et al.* 1966) and by spectrophotometric methods in a Beckman DB spectrophotometer (Nabney & Nesbitt, 1965). Binding of aflatoxin B_1 to organisms and membranes was done by incubating the cellular components with the toxin in a defined medium containing (w/v); yeast nitrogen base (Difco) 0·7 %; glucose, 0·5 % potassium phosphate 0·1 %; medium adjusted to pH 6·8. Following incubation,

the organisms and membranes were centrifuged down at 15,000 *g* for 30 min., and the concentration of toxin in the supernatant fluid determined. Washing was done by resuspending organisms or membranes in 70 ml. water followed by centrifugation.

Removal of nucleic acid from protoplast membranes. Protoplasts from $2{\cdot}0 \times 10^{11}$ bacilli were prepared and lysed, and then washed twice with 70 ml. tris buffer (pH 7·6). Membranes were resuspended in 50 ml. tris buffer (pH 7·6) + 0·5% (w/v) sodium deoxycholate and gently stirred for 1 hr. The membranes were then centrifuged down (15,000 *g* for 30 min.) and washed twice with 70 ml. tris buffer (pH 7·6) containing 10 μg deoxyribonuclease (DNase)/ml. (Sigma Chemical Co., 3500 DeKalb Street, St Louis, Mo.) and 25 μg. ribonuclease (RNase)/ml. (Sigma Chemical Co.) and gently stirred for 1 hr. The nucleic-acid-cleared membranes were harvested by centrifugation and washed twice in 70 ml. tris buffer (pH 7·6). A sample of protoplasts and membranes was sedimented, washed and extracted with perchloric acid for nucleic acid determination (Berrah & Konetzka, 1962). DNA was determined by the diphenylamine technique (Burton, 1956) and RNA by the orcinol method (Mejbaum, 1939). Weights of protoplasts and membranes were determined after drying in an air oven for 12 hr at 100°.

RESULTS

Aflatoxin inhibition of growing bacilli

Growth of *Bacillus megaterium* was inhibited in the presence of aflatoxin B_1 (Fig. 1). After a 12 hr incubation, aflatoxin B_1 at 10 and 5 μg./ml. inhibited growth 97% and 95%, respectively; at 2·5 and 1·0 μg./ml. there was no inhibition; the bacilli in a 2·5 μg./ml. concentration, however, showed a growth lag. The effects of a range of concentrations of aflatoxin B_1 on viability of growing bacilli are presented in Fig. 2. There was a striking decrease in viability of bacilli in a growth medium containing aflatoxin B_1 at 25 and 50 μg./ml., with less effect at 5·0 and 2·5 μg./ml. During a 4 hr incubation, viable bacilli decreased in the presence of aflatoxin B_1 at 50 μg./ml. from $2{\cdot}3 \times 10^6$ viable bacilli/ml. to $8{\cdot}0 \times 10^3$/ml. These tests showed that the viability of the bacilli in a growth medium containing aflatoxin at 25 or 50 μg./ml. continued to decrease during the time-course of the experiment.

Comparison of the inhibition of growth (Fig. 1) with the decrease in viability (Fig. 2) showed that aflatoxin B_1 at 5 μg./ml. did not entirely inhibit growth during the first 4 hr, whereas the viability decreased in this concentration of toxin. This difference in sensitivity to toxin was probably due to the use of growing bacilli as the inoculum in growth tests, and of non-multiplying bacilli for the inoculum in viability studies. Further examination of the aflatoxin B_1 effect on the viability of *B. megaterium* was carried out by incubation of the cells in the presence of the toxin in non-growing situations (Table 1). After a 4 hr incubation period, comparisons were made of the viability of bacilli under various conditions. Bacilli incubated at 30° aerobically demonstrated a marked decline in viability with aflatoxin B_1 at 25 and 50 μg./ml. The decrease was similar to that observed in the previous experiments (Fig. 2) and involved approximately three orders of magnitude ($3{\cdot}8 \times 10^7$ to $5{\cdot}0 \times 10^4$ bacilli). However, viability of bacilli either in nitrogen, at 5°, or with 5 μg. tetracycline/ml. was decreased less than one order of magnitude ($3{\cdot}8 \times 10^7$ to $2{\cdot}5 \times 10^7$). Evidently, the bactericidal action of aflatoxin B_1 was associated with growth of the organism.

Aflatoxin binding by intact bacilli

The nature of the binding of aflatoxin B_1 to *Bacillus megaterium* bacilli was investigated by incubating the organisms with toxin, followed by determining the concentration of toxin associated with the organisms. Of the 950 μg. of aflatoxin B_1 initially present in the incubation mixture, 525 μg. were associated with $3{\cdot}0 \times 10^{11}$ bacilli after the first centrifugation (Table 2). After the bacilli were washed 5 times

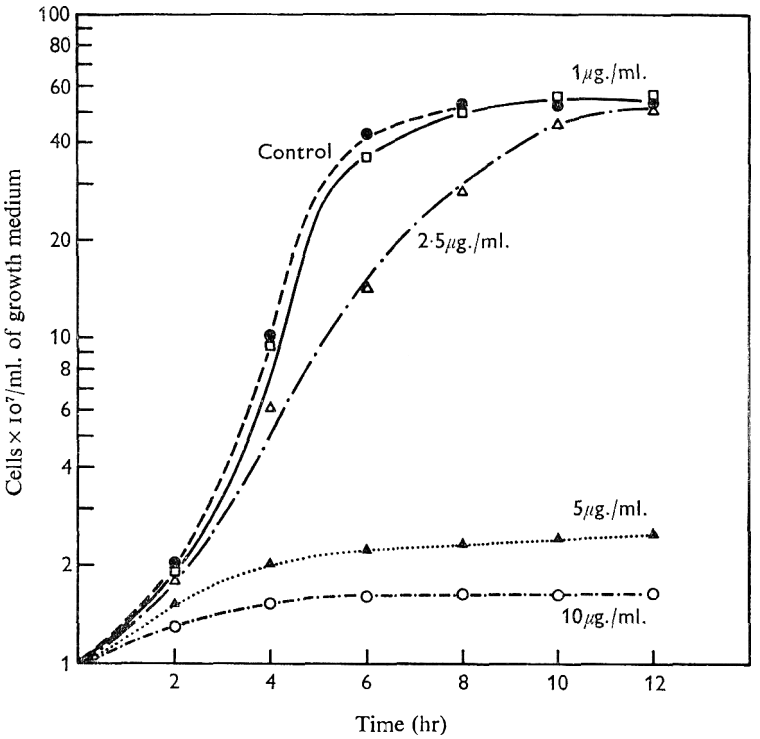

Fig. 1. Inhibition of growth of *Bacillus megaterium* by aflatoxin B_1. Ten ml. of malt-extract + yeast-extract medium (MY) inoculated with $1{\cdot}0 \times 10^8$ growing bacilli followed by incubation on a rotary shaker at 30°. Growth was measured by determining the extinction of a fixed volume of culture. Points are means of two independent observations.

with 70 ml. water/wash, 118 μg. (22%) of the 525 μg. of originally bound toxin remained associated with the bacilli. Since the decrease in bound aflatoxin B_1 between the fourth and fifth washes was only 3%, it was concluded that the 22% which remained showed a tighter attachment to the organism than did the 78% removed by the five washes. After the fifth wash, the bacilli were broken by ultrasonic treatment and extracted with chloroform. By this procedure the 'tightly bound' toxin was quantitatively recovered from the ruptured organisms.

Recovery of aflatoxin B_1 from protoplasts and protoplast membranes

Since the experiments with whole bacilli showed that tightly bound aflatoxin B_1 could be recovered from broken cells, further tests were made to elucidate the function of the cell wall in toxin-binding. Initial experiments involved measuring uptake of

aflatoxin by intact bacilli, followed by determination of toxin retention after washing, formation of protoplasts, lysing of the protoplasts and extraction of the protoplast membranes with chloroform (Table 3). After two washes, 23% of the initially bound toxin was released during the formation of protoplasts and another 7% after lysis of protoplasts. The membranes from ruptured protoplasts retained 11% of the toxin

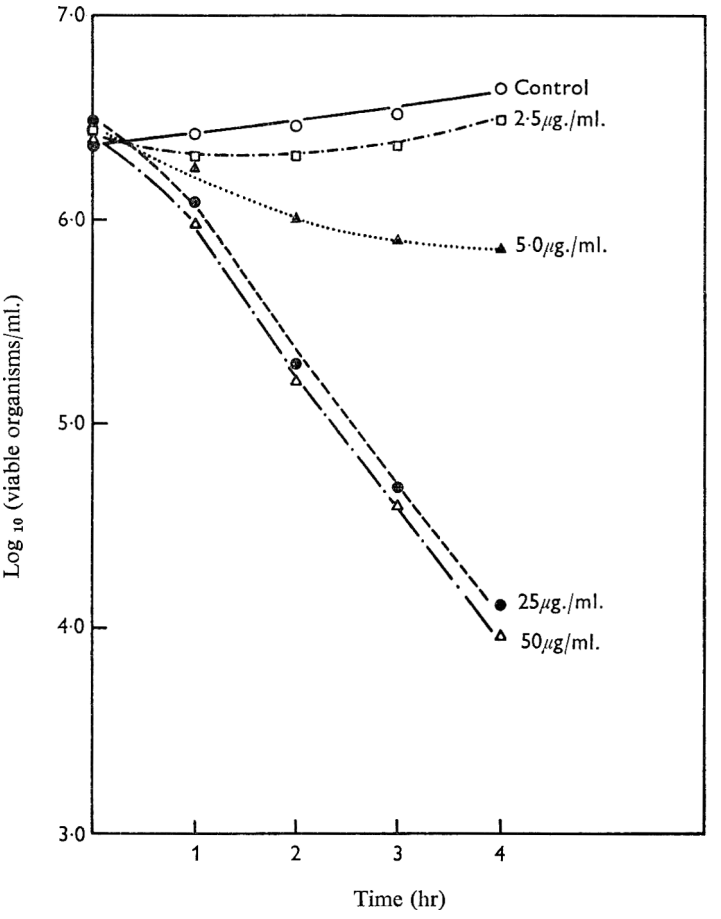

Fig. 2. Effect of various concentrations of aflatoxin B_1 on viability of growing *Bacillus megaterium*. Ten ml. of MY medium inoculated with non-multiplying bacilli followed by incubation on a rotary shaker at 30°. Samples from appropriate dilutions spread on MY agar. Points are means of three observations.

originally bound; almost all of this was recovered by chloroform extraction. These experiments showed, in general, that digestion of the cell wall by lysozyme and subsequent lysis of the protoplasts released some of the bound toxin. The 11% of toxin retained by the bacterial membranes could be largely recovered by chloroform extraction.

Since it had been clearly established that intact bacilli bind aflatoxin B_1, the question arose whether protoplasts were also capable of taking up toxin. Incubation of intact protoplasts showed that removal of the cell wall had little effect on the capacity of the organism to bind toxin (Table 4). After the protoplasts were washed and the

membranes lysed and washed, 10% of the toxin remained bound. Since washing the membranes removed only 1% of the originally bound toxin, the 10% fraction of bound toxin must have been closely associated with a membrane constituent.

Table 1. *Viability of Bacillus megaterium exposed to aflatoxin B_1*

Bacilli from a 24 hr culture (stationary phase) used as inoculum in 10 ml. MY growth medium. Flasks were incubated on a reciprocal shaker for 4 hr after inoculation. After proper dilutions, organisms plated on three MY plates, incubated for 24 hr at 28° and counted. Values given are means of the three plates for each treatment.

			Aflatoxin B_1 (μg./ml.)			
		Zero time	0	10	25	50
			Viable organisms/ml.			
Expt.	Conditions					
1	30°, air	$3{\cdot}8 \times 10^7$	$5{\cdot}6 \times 10^7$	$5{\cdot}0 \times 10^6$	$1{\cdot}5 \times 10^5$	$5{\cdot}0 \times 10^4$
	30°, N_2	$3{\cdot}8 \times 10^7$	$2{\cdot}8 \times 10^7$	$2{\cdot}7 \times 10^7$	$2{\cdot}5 \times 10^7$	$2{\cdot}5 \times 10^7$
2	30°, air	$4{\cdot}0 \times 10^7$	$7{\cdot}6 \times 10^7$	$6{\cdot}2 \times 10^6$	$1{\cdot}7 \times 10^5$	$6{\cdot}0 \times 10^4$
	5°, air	$4{\cdot}0 \times 10^7$	$2{\cdot}9 \times 10^7$	$2{\cdot}1 \times 10^7$	$2{\cdot}0 \times 10^7$	$1{\cdot}8 \times 10^7$
3	30°, air	$8{\cdot}0 \times 10^6$	$1{\cdot}2 \times 10^7$	$1{\cdot}0 \times 10^6$	$3{\cdot}8 \times 10^4$	$1{\cdot}4 \times 10^4$
	30°, air, and 5 μg./ml. tetracycline	$8{\cdot}0 \times 10^6$	$5{\cdot}0 \times 10^6$	$5{\cdot}0 \times 10^6$	$4{\cdot}0 \times 10^6$	$3{\cdot}0 \times 10^6$

Table 2. *Bacillus megaterium: recovery of bound aflatoxin B_1 from whole organisms*

$3{\cdot}0 \times 10^{11}$ bacilli were incubated with 950 μg. aflatoxin B_1 for 30 min. in 75 ml. defined growth medium at 30° on a reciprocal shaker. Values are means of two experiments.

	Aflatoxin B_1		
Treatment	Associated with bacilli, total (μg.)	μg./bacillus	% retained by bacilli
Cells from incubation	525	$1{\cdot}7 \times 10^{-9}$	100
Wash 1	330	$1{\cdot}1 \times 10^{-9}$	63
Wash 2	228	$0{\cdot}8 \times 10^{-9}$	43
Wash 3	166	$0{\cdot}6 \times 10^{-9}$	32
Wash 4	133	$0{\cdot}5 \times 10^{-9}$	25
Wash 5	118	$0{\cdot}4 \times 10^{-9}$	22
Cells treated ultrasonically and then extracted with chloroform	0	0	0

Effect of removal of nucleic acid from bacterial membranes on aflatoxin B_1 uptake

Although aflatoxin B_1 binds to nucleic acids and to proteins, the biological effects of the toxin are generally attributed to nucleic-acid binding. These observations suggested that binding of toxin to bacilli, protoplasts and membranes of *Bacillus megaterium* might be related to association with the nucleic acids. This question was explored by comparing the aflatoxin binding of control membranes to that of membranes whose nucleic acids had been removed. Table 5 presents results from treatment of the membranes with sodium deoxycholate, DNase and RNase. This procedure essentially freed the membranes of nucleic acid. The control and nucleic-acid-free

membranes did not differ significantly in their toxin-binding capacity (Table 6). It was concluded that nucleic acids are not necessary for binding of the aflatoxin B_1 to *B. megaterium* membranes.

Table 3. *Bacillus megaterium: recovery of bound aflatoxin B_1 from bacilli following formation of protoplasts*

$2{\cdot}0 \times 10^{11}$ bacilli were incubated with 1090 μg. aflatoxin B_1 for 15 min. in 50 ml. defined growth medium at 30° on a reciprocal shaker. Values are means of three experiments.

	Aflatoxin B_1		
Treatment	Associated with bacilli or membranes, total (μg.)	μg./bacillus	% retained by bacilli or membranes
Bacilli from incubation	352	$1{\cdot}8 \times 10^{-9}$	100
Wash 1	214	$1{\cdot}1 \times 10^{-9}$	61
Wash 2	143	$0{\cdot}7 \times 10^{-9}$	41
Protoplasts	63	—	18
Ruptured protoplasts	40	—	11
Ruptured protoplasts chloroform-extracted	3	—	1

Table 4. *Bacillus megaterium: recovery of bound aflatoxin B_1 from protoplasts and protoplast membranes*

Protoplasts from $2{\cdot}0 \times 10^{11}$ bacilli were incubated with 900 μg. aflatoxin B_1 for 15 min. in 50 ml. defined growth medium at 30° on a reciprocal shaker. Protoplasts were washed in 70 ml. 0·5 M-NaCl and membranes washed with 70 ml. water. Values are means of three experiments.

	Aflatoxin B_1 bound to protoplasts or membranes	
Treatment	Total (μg.)	(%)
Protoplasts from incubation	315	100
Protoplast wash 1	162	51
Protoplast wash 2	96	30
Lysed protoplasts	34	11
Membrane wash	32	10
Membranes chloroform-extracted	1	—

Table 5. *Bacillus megaterium: nucleic acid composition of protoplast and protoplast membranes*

Samples of protoplasts and membranes were sedimented, washed and extracted with perchloric acid followed by determination of DNA (diphenylamine assay) and RNA (orcinol technique). Values are means of three experiments and are based on dry weights of protoplasts and membranes.

Treatment	DNA (mg./g.)	RNA (mg./g.)
Intact protoplasts	12	122
Protoplast membranes treated deoxycholate, DNase and RNase	1	4

Table 6. *Bacillus megaterium: recovery of bound aflatoxin B_1 from protoplast membranes*

Membranes from lysed protoplasts of $2{\cdot}0 \times 10^{11}$ bacilli incubated with 900 μg. aflatoxin B_1 for 15 min. in 50 ml. defined growth medium at 30° on a reciprocal shaker. Membranes washed with 70 ml. water. Values are means of three experiments.

	Aflatoxin B_1 bound to protoplast membranes			
	With nucleic acids		Without nucleic acids	
Treatment	(μg.)	(%)	(μg.)	(%)
Membranes from incubation	301	100	305	100
Membrane wash 1	149	50	146	48
Membrane wash 2	71	24	68	22
Membrane wash 3	27	9	33	11
Membranes chloroform-extracted	0	0	2	1

DISCUSSION

Initial experiments on binding aflatoxin B_1 to intact bacilli of *Bacillus megaterium* showed that 78 % of the toxin initially bound per bacillus could be removed by aqueous washing. The remainder of the toxin associated with the organisms appeared to be more rigorously bound. However, chloroform extraction of the organisms ruptured by ultrasonic treatment gave quantitative recovery of toxin. These tests showed that although there was uptake of aflatoxin B_1 by the bacilli, there was no significant metabolic degradation of the molecule, since all the toxin was recovered. Furthermore, chloroform-extractability of the tightly bound toxin suggested that it was not covalently bound to a cellular constituent. These observations support the hypothesis of Clifford & Rees (1966, 1967) that the primary cytotoxic effect of aflatoxin B_1 involves a weak binding of the toxic molecule to a cellular constituent.

Earlier studies in our laboratory had shown that aflatoxin B_1 binds to bacterial cell walls. When whole bacilli containing toxin were converted to protoplasts 23 % of the toxin bound initially was released during digestion of the cell wall by lysozyme, and 18 % was retained by the intact protoplasts. Lysis of the protoplasts released an additional 7 %, whereas the membranes retained 11 %. These tests showed that removal of the wall did not release all the bound toxin. It was concluded that binding does not depend exclusively on the cell wall. Studies of the permeability of the protoplast to aflatoxin B_1 showed that the removal of cell wall did not prevent toxin uptake. Furthermore, following lysis of the protoplasts containing bound aflatoxin B_1, the water-washed membranes still retained 10 % of the initially bound toxin B_1. This showed that, as in intact bacilli, a portion of the bound toxin was readily dissociated from cellular constituents by aqueous washing. Examination of binding of aflatoxin B_1 by membranes, with or without nucleic acid, showed that association with DNA or RNA was not required for toxin uptake. Since a binding of toxin to protein has been reported, it seems reasonable to assume that the aflatoxin B_1 may be bound to the protein in the membranes of *Bacillus megaterium*.

The multiplying bacilli of *Bacillus megaterium* showed a marked decrease in viability in relation to bacilli in a non-multiplying situation. In some instances, toxicity of this

type has been attributed to 'unbalanced synthesis' (Roberts, McQuillen & Roberts, 1959). A similar imbalance has been observed (Lillehoj & Ciegler, 1967) in macromolecular biosynthesis of *Flavobacterium aurantiacum* grown in the presence of aflatoxin B_1 at 50 μg./ml., which decreased DNA synthesis by 80%, RNA production by 48% and protein synthesis by 32%. It appears that the imbalance in macromolecular synthesis, particularly DNA production, might be responsible for the decrease in viability of multiplying *Bacillus megaterium*.

Flavobacterium aurantiacum grown in the presence of aflatoxin B_1 develops aberrant forms (Lillehoj *et al.* 1967). *Bacillus megaterium* grown in presence of the toxin also showed an inhibition of the cell-division mechanism, and unusually long forms were routinely observed. That the toxin-induced aberrancy of *F. aurantiacum* was a manifestation of cell-wall inhibition was rejected when it was found that growth of an L-form of an aflatoxin-sensitive Bacillus strain was inhibited by the toxin (Burmeister & Hesseltine, 1967). Since the mechanism of cytokinesis in bacteria probably involves an interaction between nucleus and cell membrane (Jacob, Brenner & Cuzin, 1963), the mode of aflatoxin action in inducing aberrant forms might be due to an initial toxic event involving nucleic acid synthesis. These observations suggest to us that the intact aflatoxin B_1 molecule interferes with nucleic acid synthesis, without covalent attachment to a cellular component or metabolic degradation. Whether the binding of aflatoxin B_1 to *B. megaterium* is related to the mechanism of toxic action, however, is not clear.

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Studies on the Cell Walls of Pseudomonas Species Resistant to Ethylenediaminetetra-acetic Acid

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SUMMARY

Cell walls were prepared from various species of the genus *Pseudomonas* which are resistant to ethylenediaminetetra-acetic acid (EDTA). The cell walls were analysed and comparisons made with the walls of EDTA-sensitive pseudomonads. The walls had none of the structural features which appeared to characterize EDTA-sensitive pseudomonads. Lipopolysaccharide was not extracted from the walls of resistant organisms by EDTA at pH 9·2. The wall of *P. iodinum* contained almost no lipid or protein but consisted mainly of glycosaminopeptide and material which resembled a teichoic acid. It is proposed that this organism be removed from the genus *Pseudomonas*. The walls of *P. diminuta*, *P. maltophilia*, *P. pavonacea* and *P. rubescens* had compositions broadly characteristic of Gram-negative bacteria. The four species are not obviously related. Glycolipids were present in the walls of *P. diminuta*, *P. maltophilia* and *P. rubescens*. An ornithine-containing lipid was isolated from *P. rubescens* and partly characterized. A small amount of this lipid was also present in *P. maltophilia*. The wall of *P. maltophilia* was distinctive in its wide range of monosaccharide components, including an unidentified neutral sugar of high mobility on paper chromatograms.

INTRODUCTION

Ethylenediaminetetra-acetic acid (EDTA) has a toxic effect on *Pseudomonas aeruginosa* (MacGregor & Elliker, 1958; Gray & Wilkinson, 1965*a*; Eagon & Carson, 1965; Wilkinson, 1967), apparently the result of a lytic action by it on the cell wall of the organism (Gray & Wilkinson, 1965*a*, *b*; Eagon & Carson, 1965). A correlation has been found between bactericidal activity and chelate stability constants for polyaminocarboxylic acids related to EDTA, which indicated that chelation was involved in the toxic action of these compounds (Gray & Wilkinson, 1965*a*). The structural importance of multivalent metal cations in the wall of *P. aeruginosa* has been confirmed (Eagon & Carson, 1965; Asbell & Eagon, 1966*a*, *b*). Treatment of the bacteria with EDTA caused these cations to become soluble (Eagon & Carson, 1965) and also of the lipopolysaccharide component of the cell wall (Gray & Wilkinson, 1965*b*). Although EDTA has been found to extract lipopolysaccharide from *Escherichia coli*, the bacteria remained viable under the conditions used (Leive, 1965). Except when used in conjunction with organic cations (Wolin, 1966; Goldschmidt & Wyss, 1967; Voss, 1967), EDTA does not seem to be highly toxic for Gram-negative bacteria other than pseudomonads (Gray & Wilkinson, 1965*a*; Wilkinson, 1967). This hypersensitivity to EDTA might prove to be a useful characteristic in the taxonomy of the pseudomonads (Shively & Hartsell, 1964; Wilkinson, 1967).

In a survey of various pseudomonads (Wilkinson, 1967), only strains of the following species were found to be resistant to EDTA: *Pseudomonas diminuta*, *P. geniculata*, *P. iodinum*, *P. maltophilia*, *P. pavonacea*, *P. rubescens*. The inclusion of these organisms in the genus *Pseudomonas* has been considered doubtful by other workers who used different criteria. The cell walls of the EDTA-sensitive organisms *P. aeruginosa* and *P. alcaligenes* (originally described as *Alcaligenes faecalis*) are characterized by a relatively high content of phosphorus and have certain monosaccharide components in common, including glucose, rhamnose, glucosamine and galactosamine (Gray & Wilkinson, 1965*b*). These sugars are present in the lipopolysaccharide fractions of the walls (Clarke, Gray & Reaveley, 1967; B. A. Key, G. W. Gray & S. G. Wilkinson, unpublished observations). It was therefore decided to prepare and analyse cell walls of EDTA-resistant pseudomonads in order to verify this description of them, and in order to examine their relationship to EDTA-sensitive pseudomonads.

METHODS

Preparation of cell walls. Cell walls were prepared from *Pseudomonas diminuta* (NCTC 8545), *P. maltophilia* (NCTC 10257), *P. iodinum* (NCTC 9742), *P. pavonacea* (NCIB 9395) and *P. rubescens* (NCIB 8768). Attempts to prepare walls from *P. geniculata* (NCIB 9428) were unsuccessful as the aggregated bacteria obtained were resistant to the disintegration technique used. Organisms were grown for 24 hr on nutrient agar (Oxoid) at 25° (37° for *P. iodinum* and *P. maltophilia*). Suspensions of washed bacteria were cooled to 0° and shaken for 3 min. with acid-washed ballotini beads (no. 12) in an MSK Homogeniser (B. Braun, Melsungen, Germany). The temperature was kept below 5° by intermittent cooling with liquid CO_2. After removal of beads by filtration, the crude walls were collected and purified as described previously (Gray & Wilkinson, 1965*a*). After freeze-drying the walls were stored at 4° and were freshly dried in vacuum over P_2O_5 before quantitative analysis.

General methods. Samples of walls were examined with a Siemens Elmiskope 1a electron microscope as preparations on carbon films and were shadowed with gold palladium at a 45° angle. Equipment for high and low voltage paper electrophoresis was obtained from the Shandon Scientific Co. Ltd. A Perkin-Elmer F 11 Gas Chromatograph fitted with a flame ionization detector was used for gas–liquid chromatography. Infrared spectra were recorded by using a Unicam SP. 200 spectrophotometer; samples were normally dispersed in discs of KCl.

Solvents for paper chromatography. The following solvent systems were used for paper chromatography of water-soluble products obtained after hydrolysis of cell walls and wall fractions: I, the upper phase of ethyl acetate + pyridine + water (5 + 2 + 5, by vol.); II, the upper phase of *n*-butanol + ethanol + water + aq. ammonia sp.gr. 0·88 (40 + 10 + 49 + 1, by vol.); III, the upper phase of ethyl acetate + acetic acid + water (3 + 1 + 3, by vol.); IV, ethyl acetate + pyridine + water + acetic acid (5 + 5 + 3 + 1, by vol; Fischer & Nebel, 1955); V, ethyl acetate + pyridine + *n*-butanol + *n*-butyric acid + water (10 + 10 + 5 + 1 + 5, by vol.; Mukerjee & Ram, 1964); VI, *sec*-butanol + 88% formic acid + water (75 + 15 + 10, by vol.); VII, phenol + water + aq. ammonia sp.gr. 0·88 (80 + 20 + 1, by wt); VIII, *n*-butanol + ethanol + water (4 + 1 + 1, by vol.); IX, isopropanol + 5% aq. boric acid (7 + 1, by vol.; Ikawa, Morrow & Harney, 1966); X, *n*-propanol + aq. ammonia sp.gr. 0·88 + water (6 + 3 + 1, by vol.); XI, isopropanol +

aq. ammonia sp.gr. 0·88 + water (7 + 1 + 2, by vol.); XII, *n*-butanol + pyridine + water (6 + 4 + 3, by vol.); XIII, ethyl methyl ketone + acetic acid + water (8 + 2 + 1, by vol.); XIV, *n*-butanol + propionic acid + water (141 + 71 + 100, by vol.; Maruo & Benson, 1959); XV, *n*-butanol + acetic acid + water (5 + 1 + 2, by vol.).

Quantitative analyses. Depending on the amount of material available, phosphorus was estimated by the method of Bartlett (1959) or by the method of Allen (1940). Nitrogen was estimated by nesslerization (Umbreit, Burris & Stauffer, 1957) after digestion of samples for 16 hr with $H_2SO_4 + CuSeO_3$. Total carbohydrate was estimated by the method of Dubois *et al.* (1956) without prior hydrolysis of samples, and was expressed as glucose. Reactions with cysteine were used to estimate rhamnose (Dische & Shettles, 1948) and aldoheptoses (Osborn, 1963). Cell walls were examined for the presence of 2-keto-3-deoxyaldonic acids as described by Osborn (1963): for the purpose of calibration 0·01 μmole of acid was taken to give an extinction of 0·2 (in a 1 cm. cell) at 548 mμ. Amino compounds in hydrolysates were estimated using a Technicon AutoAnalyser. Ornithine was estimated by the acid ninhydrin reaction using the reagent (*b*) described by Work (1957).

Identification of amino acids. Samples to be analysed were hydrolysed under nitrogen in sealed ampoules with 6 N-HCl for 16 hr at 105°. Insoluble material was removed by filtration through sintered glass (no. 4 porosity) and acid was removed by repeated drying in vacuum over P_2O_5 and KOH. Amino acids in the hydrolysates were identified by ion-exchange chromatography by using the AutoAnalyser, by ascending two-dimensional paper chromatography on Whatman no. 20 paper using the solvent systems VI followed by VII, and by two-dimensional thin-layer chromatography on silica gel G (Merck) using the solvent systems chloroform + methanol + 17% (w/v) aq. ammonia (2 + 2 + 1, by vol.) followed by phenol + water (3 + 1, by wt) containing 0·02% (w/v) NaCN (Brenner, Niederwieser & Pataki, 1964). Spots were detected with ninhydrin. Ornithine was also identified by the spectrum of the product formed in the acid ninhydrin reaction, and by one-dimensional thin-layer chromatography on ether-washed layers of MN 300 cellulose (Macherey, Nagel & Co., Düren, Germany) using the solvent systems phenol + water (100 + 39, by wt) and methanol + water + pyridine + conc. HCl (32 + 7 + 4 + 1, by vol.; Perkins & Cummins, 1964).

Identification of amino sugars. Hydrolysates prepared for the analysis of amino acids or neutral sugars were sometimes examined for the presence of amino sugars. In more critical studies samples were hydrolysed for 3 hr at 105° with 4 N-HCl and the hydrolysates were neutralized using Dowex 2 resin in the bicarbonate form. The hydrolysates were then passed down short columns of Dowex 50 resin (200–400 mesh) in the hydrogen form (Boas, 1953), and the resin was washed free from neutral solutes. Amino sugars were recovered from the resin by elution with 2 N-HCl and the acid was removed by repeated drying in vacuum over P_2O_5 and KOH at room temperature. Amino sugars were identified by ion-exchange chromatography by using the AutoAnalyser. Paper chromatography was done on Whatman no. 1 paper using solvent systems IV and V (descending). Spots were detected using alkaline $AgNO_3$ (Trevelyan, Proctor & Harrison, 1950). Confirmation of glucosamine and galactosamine was obtained by ninhydrin degradation after one-dimensional chromatography in solvent system IV, followed by chromatography of the resulting pentoses in the second dimension using solvent system VIII (Stoffyn & Jeanloz, 1954). For the

identification of amino sugars by gas–liquid chromatography, their trimethylsilyl derivatives were prepared by dissolving samples containing about 200 μg. total amino sugar in dry *N,N*-dimethylformamide (60 μl.) and adding bis-(trimethylsilyl)-acetamide (40 μl.). After about 1 hr at room temperature, samples were analysed using a column packed with 15% (w/w) Apiezon M on Chromosorb W (60–80 mesh) and operated at 225° with a nitrogen flow rate of 27 ml./min.

Identification of sugars. Samples were hydrolysed for 2 hr at 105° with 2 N-HCl, and the hydrolysates neutralized with Dowex 2 resin in the bicarbonate form. When necessary, neutral sugars were separated from amino acids and amino sugars by passage of hydrolysates down cation-exchange columns as described above. Descending paper chromatography was done on Whatman no. 1 paper, usually using the solvent systems I, II, III and IV, detecting spots with alkaline $AgNO_3$, aniline hydrogen phthalate and periodate-Schiff's reagents (Baddiley, Buchanan, Handschumacher & Prescott, 1956). For special purposes the solvent systems VIII, IX, XII, XIII and XV and the detection reagents periodate–thiobarbiturate (Warren, 1960; Cynkin & Ashwell, 1960), *p*-anisidine hydrochloride, naphthoresorcinol (Bryson & Mitchell, 1951), periodate-*p*-nitroaniline and *p*-*N,N*-dimethylaminobenzaldchyde-HCl (Edward & Waldron, 1952) were used. When necessary sugars were eluted from chromatograms and their identities confirmed by further tests. Xylose was conclusively differentiated from lyxose by low voltage electrophoresis at 0·4 mA/cm. for 4 hr in 0·05 M-borate buffer (pH 9·6), and also by gas–liquid chromatography of its trimethylsilyl derivative (Sweeley, Bentley, Makita & Wells, 1963). Chromatography was done using a column of 20% (w/w) silicone SE-30 on Chromosorb W (60–80 mesh) operated at 175° with a nitrogen flow rate of 20 ml./min.

Extraction of loosely bound lipids. Samples (10–25 mg.) of walls were stirred under nitrogen with chloroform + methanol (2 + 1, by vol.; 5 ml.) for 2 hr at room temperature. Insoluble residues were collected by filtration of the extract through sintered glass (no. 4 porosity) and were washed with further solvent (10 ml.). The combined extracts and washings were collected in tared weighing bottles and the solvent was removed by evaporation under a stream of nitrogen at room temperature. The lipid residues were dried overnight in vacuum over P_2O_5 and, after weighing, were dissolved in chloroform + methanol and stored under nitrogen at $-20°$. Further extractions on a larger scale were made with walls of *Pseudomonas diminuta*, *P. iodinum* and *P. maltophilia*, and with freeze-dried whole *P. rubescens* organisms.

Thin-layer chromatography of lipids. For the identification of phospholipids and other polar lipids, samples were normally examined by chromatography on silica gel G using chloroform + methanol + water (65 + 25 + 4, by vol.). Further information was obtained with the solvent system chloroform + methanol + 7 N-aq. ammonia (65 + 25 + 4, by vol.; Nichols, 1964), with silica gel H and the Systems I (two-step) and III of Skipski, Barclay, Reichman & Good (1967), and with basic silica gel H and chloroform + methanol + acetic acid + water (50 + 25 + 8 + 4, by vol.; Skipski, Peterson, Sanders & Barclay, 1963). Spots were detected using iodine vapour, ninhydrin, periodate–Schiff's reagents and reagents for phosphorus (Wagner, Hörhammer & Wolff, 1961; Dittmer & Lester, 1964). Samples were also examined for the presence of neutral lipids and fatty acids by the method of Skipski, Smolowe, Sullivan & Barclay (1965); spots were detected by iodine vapour and by a modified H_2SO_4 spray (Zimiński & Borowski, 1966).

Fractionation of lipids from walls of Pseudomonas diminuta. A sample of total lipids (17.89 mg.) was dissolved in chloroform (4 ml.) and the solution was clarified by filtration through sintered glass (no. 4 porosity). Ether (16 ml.) was added to the filtrate and the flocculent white precipitate which developed on standing was collected by filtration, washed with ether and redissolved in chloroform. The weights of lipid soluble and insoluble in ethereal chloroform were 14·41 and 2·87 mg., respectively. The composition of the soluble fraction was studied by thin-layer chromatography. Glycolipids present were isolated by elution from appropriate zones of silica and were partly characterized as described elsewhere (Wilkinson, 1968).

Fractionation of lipids from walls of Pseudomonas maltophilia. A sample of total lipids (26 mg.) was fractionated on a column of silicic acid (Mallinckrodt, 100 mesh; 7 g.) packed in chloroform, by stepwise elution with chloroform (100 ml.) and 5, 10, 20 and 33% (v/v) solutions of methanol in chloroform (75 ml. each). Fractions of 5 ml. were collected and samples (1%) were screened by thin-layer chromatography. Samples of appropriately bulked fractions were retained and the remaining material was recombined and subjected to further chromatography on a similar column of different silicic acid (Baker's Analysed Reagent) using the same scheme of elution. Fractions were again screened by thin-layer chromatography and also by identification of the products formed on mild alkaline hydrolysis of the phospholipids. The lipid samples (containing up to 300 μg. phosphorus) were dissolved in chloroform (1 ml.) and 0·2 N-methanolic KOH (1 ml.) was added. After incubation of the mixture for 20 min. at 37°, ethyl formate (0·05 ml.) was added and incubation was continued for 5 min. Chloroform (1 ml.) and water (1 ml.) were added and the layers were thoroughly mixed. After centrifugation the aqueous layer was withdrawn and the chloroform layer was further extracted with water (2 × 1 ml.). With the total phospholipids from *P. maltophilia* only 1·3% of the initial phosphorus remained in the chloroform layer. The combined aqueous extracts were passed down a column of Dowex 50 in the ammonium form and the eluate was dried in vacuum over P_2O_5. The residue was examined by paper chromatography using solvent systems X and XIV (ascending) on Whatman no. 541 paper and system XI (descending) on Whatman no. 1 paper. Chromatograms were sprayed with ninhydrin, periodate-Schiff's reagents and the reagent of Hanes & Isherwood (1949) for phosphorus. The water-soluble products were also identified by high voltage electrophoresis (5000 V for 1 hr) on Whatman no. 1 paper in pyridine+acetic acid buffer (pH 3·6; Dawson, 1960). Limited tests were applied to column fractions containing glycolipids and an ornithine-containing lipid also found in *P. rubescens* (see below).

Fractionation of lipids from whole Pseudomonas rubescens organisms. The major ninhydrin-positive lipid in the cell wall of *P. rubescens* contained ornithine but no phosphorus. To obtain larger amounts of this material, lipids were extracted from whole organisms. The total lipids (95 mg., 8·2% of dry weight) were fractionated on a column of silicic acid (Mallinckrodt) as described for *P. maltophilia*. A mixture of two glycolipids also present in the cell wall was eluted with 5% (v/v) methanol in chloroform and was studied separately (Wilkinson, 1968). The lipid containing ornithine was eluted in late fractions of 10% (v/v) methanol and in 20% (v/v) methanol in chloroform. The trace of phosphatidylglycerol and most of the phosphatidylethanolamine present in the combined fractions were removed by chromatography on a column (12 cm. × 2 cm.) of DEAE-cellulose (Whatman DE 23) in the acetate form (Rouser, Kritchevsky, Heller

& Lieber, 1963). The crude ornithine-containing lipid was eluted using 12·5% (v/v) methanol in chloroform (350 ml.). After repeat chromatography on a second column of DEAE-cellulose the lipid (18·9 mg., 0·53% phosphorus) still contained about 10% phosphatidylethanolamine. The mixture (7 mg.) was subjected to mild alkaline hydrolysis (Gorchien, 1964) during which all of the phospholipid, but only part of the ornithine-containing lipid, was degraded. The intact lipid and its ninhydrin-positive degradation product were separated by chromatography on silicic acid and were purified by preparative thin-layer chromatography on silica gel G using chloroform + methanol + water (65 + 25 + 4, by vol.). Zones of silica containing the lipids were transferred to small columns and the lipids were eluted using solutions of increasing concentration of methanol in chloroform. After removal of the solvents using a rotary evaporator, the residues were dissolved in chloroform + methanol (2 + 1, by vol., 4 ml.) and the solutions were washed with water (1 ml.) to remove non-lipid contaminants (Duthie & Patton, 1965). The final yields of the intact lipid and its degradation product were 2·12 and 0·57 mg., respectively. A sample of the intact lipid was hydrolysed under nitrogen with 2 N-HCl (1 ml.) for 3 hr at 105° and the hydrolysate was extracted with light petroleum (b.p. 60–80°; 6 × 1 ml.). Fatty acids present in the extract were methylated using BF_3-methanol (Morrison & Smith, 1964). The methyl esters were examined by gas–liquid chromatography using columns of 10% (w/w) poly-diethylene glycol succinate on Chromosorb W (80–100 mesh) and 20% (w/w) Apiezon L on Chromosorb P (60–80 mesh). Oven temperatures were 160° and 220° and nitrogen flow rates were 26 and 31 ml./min. for the polar and non-polar columns, respectively.

A further sample of the intact lipid was reacted with 2,4-dinitrofluorobenzene (Wheeldon & Collins, 1957) and the DNP-lipid was hydrolysed with 6 N-HCl for 4 hr at 105°. The hydrolysate was extracted with ether (6 × 1 ml.) and the aqueous layer was dried *in vacuo* over P_2O_5 and KOH. The residue was dissolved in *n*-butanol + ethyl acetate (1 + 1, by vol.) and examined by thin-layer chromatography on silica gel G using the solvent systems *n*-propanol + aq. ammonia sp.gr. 0·88 (7 + 3 by vol.; Brenner, Niederwieser & Pataki, 1961), the upper phase of *n*-butanol + acetic acid + water (4 + 1 + 5, by vol.) and 2-chloroethanol + toluene + pyridine + 20% (w/v) aq. ammonia (10 + 7 + 3 + 2, by vol.; Pataki, 1967).

Extraction of cell walls of Pseudomonas iodinum with trichloroacetic acid. Lipid-free walls (202 mg.) of *P. iodinum* were stirred for 24 hr at 4° with 10% (w/v) aq. trichloroacetic acid (25 ml.) Insoluble material was collected by centrifugation, extracted for 48 hr with further solvent (25 ml.), washed four times with deionized water and freeze-dried (yield 119 mg.). Both extracts were clarified by filtration through glass sinters (no. 4 porosity) and each was added to acetone (100 ml.) at 4°. As very little precipitate formed on standing for 4 days at 4°, most of the acetone was removed from the combined extracts at 20° using a rotary evaporator. The aqueous residue was made to 100 ml. and samples were taken for phosphorus analysis. The remaining solution was extracted with *n*-butyl acetate (6 × 100 ml.; Young, 1964), neutralized by the addition of 2 drops of dil. aq. ammonia and freeze-dried. The white, deliquescent solid (119 mg.) was analysed for phosphorus and sugars.

As the weight of the crude extract exceeded the loss in weight of the cell walls (83 mg.), the remaining material was dissolved in water (10 ml.) and was dialysed for 3 days at 4° against deionized water (2 l.), with external stirring and four changes of water. During dialysis 78% of the weight of material and 30% of the total phosphorus

passed into the diffusate. The non-diffusable residue was dried down as a colourless glass (20 mg.). The mobility of phosphorus-containing material on low voltage (0·6 mA/cm. for 3 hr) and high voltage (5000 V for 1·5 hr) electrophoresis in pyridine + acetic acid buffer (pH 3·6), and the behaviour on paper chromatography in solvent system X were studied. Organic phosphates formed on hydrolysis with 2 N-HCl for 3 hr at 105° were eluted from a paper chromatogram run in solvent system X, and were incubated under toluene with alkaline phosphatase (Sigma Chemical Co.) at 1 mg./ml. in 0·05 M-ammonium carbonate buffer (pH 9·3) for 16 hr at 37°. After drying down, the residue was examined for sugars and polyols by paper chromatography.

Extraction of cell walls with EDTA. Suspensions of cell walls (4 mg./ml.) in 0·05 M-borate buffer (pH 9·2) were treated with equal volumes of 6·8 mM-EDTA in the buffer or of buffer alone. After 1 hr at 20° insoluble residues were deposited by centrifugation for 1 hr at 10,000 rev./min. and the supernatant fluids were analysed for phosphorus (Gray & Wilkinson, 1965*b*).

RESULTS

General observations

Although the quantitative results tabulated below relate only to single batches of cell walls from each bacterium, analyses on different batches were qualitatively and, except in the case of *Pseudomonas iodinum*, quantitatively reproduced. The nature of the analytical variations for *P. iodinum* is described in a later section. Only in preparations from *P. diminuta* was there evidence from electron micrographs of significant contamination of the walls by cytoplasmic or other materials. The preparations from *P. diminuta* contained a small amount of material similar to that found to contaminate wall preparations from *Rhizobium trifolii* (Vincent, Humphrey & North, 1962). The possibility that this material was β-hydroxybutyrate polymer was borne out by subsequent studies on lipids extracted from the preparations. The cell walls from *P. iodinum* differed notably from the others in being pure white and in the ease with which clean preparations were obtained by washing procedures alone. Although treatment with enzymes was included in the purification scheme, this step appeared to be unnecessary with *P. iodinum*. These properties and the smooth, clean appearance of the walls in electron micrographs are usually more characteristic of Gram-positive than of Gram-negative bacteria. The infrared spectrum of the walls of *P. iodinum* (Fig. 1) also differed appreciably from those of the cell walls of Gram-negative bacteria (Gray & Wilkinson, 1965*b*). The relatively weak absorptions at about 2900 $cm.^{-1}$ (associated with C–H stretching vibrations) and at 1655 and 1550 $cm.^{-1}$ (amide I and II bands) indicate that the cell walls contain little lipid or protein. The infrared spectra of the other cell walls studied are dominated by absorption bands from protein components. The spectrum of the walls of *P. diminuta* also contains an unusually strong band at 1730 $cm.^{-1}$ (probably ester carbonyl absorption). None of the spectra contain the minor absorption band at 928 $cm.^{-1}$ present in the spectra of the EDTA-sensitive organisms *P. aeruginosa* and *P. alcaligenes* (Gray & Wilkinson, 1965*b*).

General analyses

The results of quantitative analyses on the cell walls are summarized in Table 1. It can be seen that the walls of *Pseudomonas iodinum* contained essentially no lipid, while the low nitrogen analysis was also consistent with a low content of protein as inferred from the infrared spectrum. The phosphorus content of the cell walls of *P. iodinum* was notably high while that of the walls of *P. diminuta* was notably low.

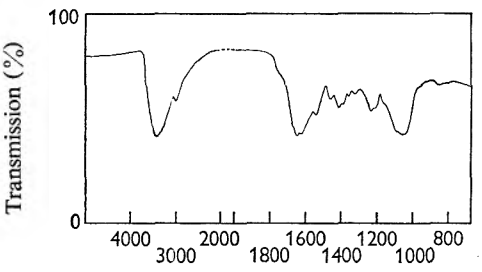

Fig. 1

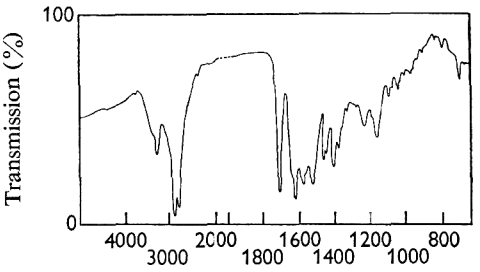

Fig. 2

Fig. 1. Infrared spectrum of cell walls of *Pseudomonas iodinum*.

Fig. 2. Infrared spectrum of ornithine-containing lipid from *Pseudomonas rubescens*. The lipid was dispersed in a KCl disc.

Amino acid composition

It was confirmed by paper, thin-layer and ion-exchange chromatography that the walls of *Pseudomonas diminuta*, *P. maltophilia*, *P. pavonacea*, and *P. rubescens* contained a wide range of amino acids in amounts expected for protein-containing materials. In the walls of *P. iodinum* only alanine, glutamic acid and α,ϵ-diaminopimelic acid (together with amino sugars) were present in major amounts. The results of quantitative analyses of amino compounds are given in Table 2; nitrogen recoveries were in the range 93–105%.

Amino sugar components

The glycosaminopeptide components muramic acid and glucosamine were present in each of the wall preparations. The results of quantitative analyses (uncorrected for destruction of amino sugars during hydrolysis) are included in Table 2. Galactosamine was a minor component of the wall of *Pseudomonas iodinum* and was not detected in the walls of the other bacteria. Direct preparation of the trimethylsilyl derivatives of the three amino sugars, without prior *N*-acetylation by the method described, was found to be more reliable than other methods reported (Kärkkäinen, Lehtonen & Nikkari, 1965; Radhakrishnamurthy, Dalferes & Berenson, 1966). By using a column of Apiezon M two peaks were found for each amino sugar. Retention times relative to that of the derivative from α-glucopyranose were: glucosamine, 0·99 and 1·22; galactosamine, 0·76 and 0·86; muramic acid, 2·32 and 2·71. No interference from amino acids present in the hydrolysates was found under the conditions used.

Table 1. *General analyses of cell walls of some Pseudomonas species*

Species	Nitrogen (%)	Phosphorus (%)	Carbohydrate* (%)	2-Keto-3-deoxyaldonic acid† (%)	Lipid (%)
P. diminuta	8·53	0·15	14·3	1·05	28·2
P. iodinum	5·10	3·07	11·3	2·04	0·4
P. maltophilia	9·74	0·79	20·8	0·22	12·7
P. pavonacea	8·29	1·09	5·4	1·01	11·1
P. rubescens	8·36	0·96	11·1	0	23·7

* Expressed as glucose and corrected for controls.
† Expressed as 2-keto-3-deoxyoctonic acid.

Table 2. *Analyses of amino compounds present in cell walls of some Pseudomonas species*

Cell walls were hydrolysed for 16 hr at 105° with 6 N-HCl. Results are calculated for residues of amino compounds and are not corrected for destruction or slow release during hydrolysis.

Compound	Organism: *P. diminuta*	*P. iodinum*	*P. maltophilia*	*P. pavonacea*	*P. rubescens*
	Component of wall (%)				
Aspartic acid	5·54	0·33	6·51	5·04	6·43
Threonine	3·04	0·15	2·44	2·22	2·89
Serine	2·50	0·30	2·70	1·83	2·95
Glutamic acid	5·43	6·82	6·04	7·02	5·14
Proline	2·48	trace	2·45	1·15	1·18
Glycine	2·69	0·19	2·45	1·60	2·10
Alanine	5·00	7·33	4·43	5·61	3·52
Valine	2·79	0·15	2·62	1·88	2·50
Cystine	0·13	trace	0·21	0·10	0·10
Methionine	0·64	trace	0·74	0·43	1·14
Isoleucine	1·50	0·05	1·56	1·36	1·46
Leucine	3·33	0·09	3·66	2·80	4·03
Tyrosine	2·29	0·12	2·74	1·97	2·68
Phenylalanine	2·66	0·07	2·65	1·68	2·92
Diaminopimelic acid	1·83	8·29	1·91	4·05	0·86
Ornithine	0·24	0·15	0·09	0·23	1·90
Lysine	1·33	0·39	2·38	1·87	2·83
Histidine	0·66	0·12	0·65	0 49	0·49
Arginine	3·43	0·16	3·70	1 58	1·89
Glucosamine	1·17	6·02	2·03	3 85	1·51
Galactosamine	0	0·64	0	0	0
Muramic acid	1·49	7·12	1·46	3 91	0·50
Ethanolamine	0	0	0·14	0 26	0·17

Sugar components

The monosaccharide components of the walls (other than amino sugars) whose presence was confirmed by chromatography are given in Table 3, which also indicates the amount of each sugar relative to other sugars in the same cell wall, as judged by the intensities of spots on paper chromatograms. In addition to the monosaccharides listed, hexuronic acids have been detected in the wall lipids of *Pseudomonas diminuta*,

P. maltophilia and *P. rubescens* (Wilkinson, 1968). Estimates for total carbohydrate and for 2-keto-3-deoxyaldonic acids (Table 1), and for rhamnose in the wall of *P. maltophilia* (16·4%), were made by colorimetric methods. In the thiobarbiturate test for 2-keto-3-deoxyaldonic acids the absorption peaks produced were as expected for *P. diminuta* (549 mμ), *P. iodinum* (548 mμ) and *P. pavonacea* (548 mμ). Under comparable conditions a weak absorption peak at 543 mμ was obtained for *P. maltophilia*, while *P. rubescens* gave no obvious reaction. In the cysteine reaction for aldoheptoses none of the walls gave spectra containing significant peaks at about 505 mμ (a minor peak at 507 mμ for *P. rubescens* was also present in a control without added cysteine). By using the differential extinction (505 mμ minus 545 mμ) given by Osborn (1963) for L-glycero-D-mannoheptose, apparent heptose contents were calculated for the walls of *P. diminuta* (0·55%), *P. maltophilia* (0·93%) and *P. rubescens* (1·15%); results for *P. iodinum* and *P. pavonacea* were negligible. Confirmation of suspected heptoses was not attempted.

Table 3. *Monosaccharide components of cell walls of some Pseudomonas species*

Monosaccharides were detected by paper chromatography. The amount of each component relative to other components of the same cell wall was judged by the size and intensity of the spot obtained, and is expressed on the arbitrary scale 0–10.

Component	*P. diminuta*	*P. iodinum*	*P. maltophilia*	*P. pavonacea*	*P. rubescens*
Glucose	5	5	3	1	5
Galactose	2	3	1	5	2
Mannose	3	0	1	0	0
Xylose	0	0	2	0	0
Rhamnose	0	0	10	0	0
Unknown	0	0	2	0	0

The unknown sugar present in the wall of *Pseudomonas maltophilia* could not be identified by paper chromatography. It was not adsorbed either by cation- or anion-exchange resins and therefore appeared to be a neutral sugar. It appeared to be fairly rapidly released on acid hydrolysis of the walls (after 5 min. at 100° in N-HCl only rhamnose was present in the hydrolysate in larger amount) and was not noticeably acid labile. It readily reduced alkaline $AgNO_3$ and its colour reactions with aniline hydrogen phthalate, *p*-anisidine hydrochloride and naphthoresorcinol were the same as for the aldopentoses. No reaction was detected with periodate-Schiff's reagents, ninhydrin, thiobarbiturate reagents or reagents for deoxysugars (Edward & Waldron, 1952) with amounts of sample which were readily detected by the preceding reagents. The R_{Rhamnose} values of the compound on descending paper chromatography in various solvent systems were as follows I, 1·19; II, 1·19; III, 1·41; IV, 1·12; XII, 1·14; XIII, 1·39; XV, 1·17. The compound was differentiated from the 3,6-dideoxyhexoses which occur in bacterial lipopolysaccharides by its relative stability to acids and by its reactions with aniline hydrogen phthalate and periodate-thiobarbiturate reagents (Westphal & Lüderitz, 1960). If the compound is a derivative of an aldopentose its high mobility on chromatograms might be explained, for example, by the presence of an *O*-methyl substituent.

Lipid components

The contents of the loosely bound lipids of the wall preparations are given in Table 1. Phosphorus analyses of the lipids were: *Pseudomonas diminuta*, 0·21 %; *P. maltophilia*, 2·34 %; *P. pavonacea*, 2·92 %; *P. rubescens*, 0·69 %. The range of components in each of the lipid extracts, as revealed by thin-layer chromatography, is illustrated in Fig. 3. Some of the components could be identified by using a variety of solvent systems and spray reagents, but others required more detailed study.

Lipids from Pseudomonas diminuta. The total lipids from *P. diminuta* were separated into an ether-soluble fraction (83·4 %) and an ether-insoluble fraction (16·6 %). The latter fraction was obtained as a translucent plastic film on drying a solution in chloroform. The infrared spectrum of the film (pressed between KBr windows) closely resembled a reference spectrum of poly-β-hydroxybutyrate, although there was evidence for a small amount of additional ester material. The film was rolled into a capillary tube and its melting range determined as 167–169°. On stronger heating the liquid decomposed and colourless crystals (presumably crotonic acid) were formed in the cooler part of the tube. Thus the original wall preparation appeared to contain about 4·7 % of poly-β-hydroxybutyrate. The polymer may correspond to the spot of highest R_F value in Fig. 3.

Of the ether-soluble lipids only two minor components contained phosphorus. The component having the higher R_F value might have been phosphatidic acid, while the other phospholipid rapidly gave a purple colour with periodate–Schiff's reagents and was probably phosphatidylglycerol. Small amounts of free fatty acid and a series of unidentified ninhydrin-positive components were also present. As lipid extracts were not treated for the removal of non-lipid contaminants, the latter may be impurities. The major ether-soluble lipids (D 2 and D 6 in Fig. 3) were isolated by thin-layer chromatography and have been partly characterized as glycolipids containing glucose and a hexuronic acid (Wilkinson, 1968).

Lipids from Pseudomonas rubescens. Glycolipids were isolated from whole cells of *P. rubescens* and were present in the wall lipids of this organism (R 3 and R 7 in Fig. 3). Small amounts of unidentified neutral lipids and fatty acids and a trace of phosphatidylglycerol were also present in the cell-wall extract, together with larger amounts of phosphatidylethanolamine and an unidentified, ninhydrin-positive phospholipid. Phosphatidylethanolamine was calculated from the ethanolamine content of the total lipid extract to constitute about 12 % of the lipid and to account for about 77 % of the lipid phosphorus. Although the shape and position of the spot for the unidentified phospholipid (R 9 in Fig. 3) resemble those of phosphatidylserine, it was readily differentiated from the latter compound using a basic plate and an acidic solvent system (Skipski *et al.* 1963). This lipid has not been studied further.

The major lipid in the wall of *Pseudomonas rubescens* reacted strongly with ninhydrin (although more slowly than did phosphatidylethanolamine) and did not contain phosphorus. It overlapped, or was barely separated from, phosphatidylethanolamine in all the solvent systems tried. In these respects it resembled the ornithine-containing lipid recently shown to occur in a number of other bacteria (Lanéelle, Lanéelle & Asselineau, 1963; Gorchein, 1964; Depinto, 1967). It was confirmed that the total lipids from the wall of *P. rubescens* contained about 8 % ornithine. Virtually

all of the ornithine present in the cell wall was removed by extraction with chloroform + methanol (2 + 1, by vol.).

A small amount of the ornithine-containing lipid was isolated from whole *Pseudomonas rubescens* organisms as described under Methods. The infrared spectrum of the purified lipid is given in Fig. 2. It contains absorption bands indicative of ester, amide and amino groups. No ester carbonyl absorption band was present in the spectrum of the partly degraded lipid isolated after alkaline hydrolysis, and absorption bands in the region 1500–1700 cm.$^{-1}$ were unresolved. The ornithine content of the intact lipid

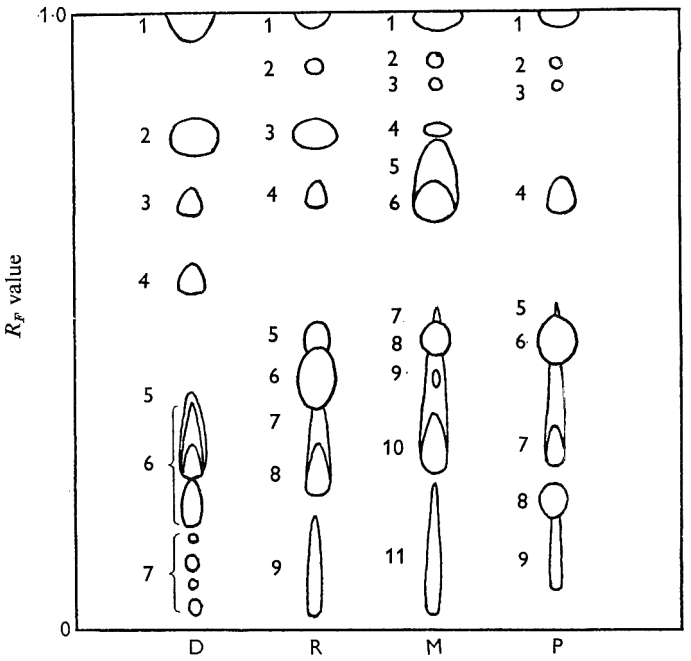

Fig. 3. Thin-layer chromatography of cell-wall extractable lipids. Drawing of a chromatogram on silica gel G developed with chloroform + methanol + water (65 + 25 + 4, by vol.). D, *Pseudomonas diminuta*: 1, neutral lipid; 2, glycolipid; 3, fatty acid; 4, phosphatidic acid (?); 5, phosphatidylglycerol; 6, glycolipids; 7, ninhydrin-positive components. R, *Pseudomonas rubescens*: 1, 2, neutral lipids; 3, glycolipid; 4, fatty acid; 5, phosphatidylethanolamine; 6, ornithine-containing lipid; 7, glycolipid; 8, phosphatidylglycerol; 9, unidentified ninhydrin-positive phospholipid. M, *Pseudomonas maltophilia*: 1, 2, 3, neutral lipids; 4, glycolipid; 5, cardiolipin; 6, fatty acid; 7, unidentified phospholipid; 8, phosphatidylethanolamine; 9, ornithine-containing lipid; 10, glycolipid and phosphatidylglycerol; 11, unidentified phospholipid. P, *Pseudomonas pavonacea*: 1, 2, 3, neutral lipids; 4, fatty acid; 5, unidentified phospholipid; 6, phosphatidylethanolamine; 7, phosphatidylglycerol: 8, lysophosphatidylethanolamine (?); 9, unidentified phospholipid.

was 21 %, compared with the value of 17 % found for the corresponding lipid from a Mycobacterium species (Lanéelle *et al.* 1963). As these authors have pointed out, these results indicate that the lipid contains two long-chain fatty residues per mole of ornithine. The analysis also indicated that the lipid constituted about 40 % of the total lipid in the cell wall of *P. rubescens*. The lipid reacted with 2,4-dinitrofluorobenzene to give on hydrolysis an acid-soluble, ninhydrin-positive DNP-derivative. The derivative moved as a single spot corresponding to δ-DNP-ornithine on thin-layer

chromatography using *n*-propanol+aq. ammonia sp.gr. 0·88 (7+3, by vol.). The results obtained with two other solvent systems were similar except that the lipid derivative was split into two closely spaced spots, whereas the reference compound was not. As ornithine was the only amino acid present in the lipid these results might be explained in terms of an ionic equilibrium; a sample of α-DNP-ornithine was not available for chromatographic comparisons.

Simple long-chain alcohols such as *n*-hexadecanol were not detected either by thin-layer or gas–liquid chromatography of material extracted by light petroleum from an acid hydrolysate of the ornithine-containing lipid, nor of the chloroform-soluble material obtained during mild alkaline hydrolysis of the crude lipid. After methylation of the material from the acid hydrolysate at least 20 components were detected by gas–liquid chromatography. Of the nine major components only the methyl esters of the straight chain C_{16}, $C_{16:1}$ and $C_{18:1}$ acids were readily recognized. Further work on this lipid will be reported separately.

Lipids from Pseudomonas maltophilia. A small amount of an ornithine-containing lipid was also present in the wall of *P. maltophilia*. It had the same properties as the corresponding lipid from *P. rubescens* both on thin-layer and silicic acid column chromatography. Small or trace amounts of glycolipids, fatty acids and neutral lipids were also present. One of the neutral lipids had the mobility of a hydrocarbon on thin-layer chromatography. Of the five phospholipids in the extract, the one present in the largest amount was apparently cardiolipin. The water-soluble phosphate ester formed on mild alkaline hydrolysis had the same properties as the corresponding product from a reference sample of cardiolipin (Sylvana Chemical Co., Orange, New Jersey, U.S.A.) on high voltage electrophoresis and on paper chromatography in three solvent systems. On thin-layer chromatograms the parent lipid moved rather more slowly than the reference compound in all solvent systems tried, and its behaviour on a silicic acid column was also uncharacteristic. Whereas cardiolipin is normally the first of the common phospholipids to be eluted, only a small amount of the lipid from *P. maltophilia* was eluted using 5 and 10% (v/v) methanol in chloroform. Most of the lipid was eluted along with phosphatidylethanolamine (M 8 in Fig. 3) using 20% (v/v) methanol, while a further amount free of phosphatidylethanolamine was only recovered using 33% (v/v) methanol in chloroform. This result was reproduced using two different grades of silicic acid. It was similarly shown that the small amount of phosphatidylglycerol present in the extract was retarded on a column compared with the same lipid from *P. rubescens*. These findings might be most easily explained by the effects of cations on the chromatographic properties of the lipids (De Haas, Bonsen & van Deenen, 1966), although the possibility of structural variations has to be considered (Courtade, Marinetti & Stotz, 1967). Of the two remaining phospholipids from *P. maltophilia* at least one (M 7 in Fig. 3) may be a decomposition product of cardiolipin; it was eluted from silicic acid using 33% (v/v) methanol in chloroform. After deacylation of a mixture of the four phospholipids of highest R_F values, only spots corresponding to bis-(glycerophosphoryl)-glycerol, glycerophosphorylglycerol and glycerophosphorylethanolamine were detected on paper chromatography and electrophoresis. The fifth phospholipid has not been further studied.

Lipids from Pseudomonas pavonacea. As only 1·16 mg. of lipid from the wall of *P. pavonacea* was obtained, extensive studies were not possible. Phosphorus analysis and thin-layer chromatography of the extract showed a high content of phospholipids,

principally phosphatidylethanolamine. A moderate amount of phosphatidylglycerol, two unidentified phospholipids and a ninhydrin-positive phospholipid having the R_F values of lysophosphatidylethanolamine were also detected. However, the possibility that the latter compound was an *O*-amino acyl ester of phosphatidylglycerol was not excluded. The other unidentified phospholipids (P 5 and P 9 in Fig. 3) resembled those described for *P. maltophilia*. Although cardiolipin was not detected, the substantial amount of the compound thought to be lysophosphatidylethanolamine suggested that degradation of the phospholipids of *P. pavonacea* might have been unusually extensive. A moderate amount of free fatty acid and traces of neutral lipids were also found in the extract.

Material extracted from cell walls of Pseudomonas iodinum by trichloroacetic acid

The Gram-positive character and the high phosphorus content of the cell wall of *P. iodinum* suggested that a teichoic acid might be present. To examine this possibility a larger quantity of cell walls was prepared. As with the first batch, the walls contained very little lipid (0·6%) and very little protein relative to glycosaminopeptide. However, the phosphorus content was reduced to 1·67% and more galactose than glucose was present. During extractions with trichloroacetic acid two-thirds of the total phosphorus was solubilised. Only 1% of the soluble phosphorus was estimated as inorganic orthophosphate, and none of it was extracted from aqueous solution by *n*-butyl acetate. The solid obtained on drying the crude extract contained both glucose and galactose but only traces of ninhydrin-reactive compounds. Several spots corresponding to organic monophosphates and diphosphates were detected on chromatography of an acid hydrolysate.

After purification by dialysis 20 mg. of non-diffusable material remained. It contained 4·32% phosphorus, 2·0% nitrogen and 31·6% carbohydrate (calculated as glucose although galactose was still the major sugar). After hydrolysis for 4 hr at 105° with 6 N-HCl, at least 80% of the nitrogen in the sample was recovered as ammonia; galactosamine (0·24%) and traces of amino acids were also found. On low voltage electrophoresis the phosphorus-containing material moved as a discrete oval spot. The distance moved by the centre of the spot after 3 hr at 0·6 mA/cm. was 5·8 cm., compared with α-glycerophosphate (4·9 cm.) and α,α′-glycerol diphosphate (6·4 cm.). On high voltage electrophoresis the material moved as a long streak. Similarly, on paper chromatography using solvent system X the material streaked forward from the origin.

After acid hydrolysis, glycerol and possibly glycerophosphate were detected; ribitol, anhydroribitol and inositol were absent. An unidentified compound was also detected in the hydrolysate. The compound gave a weak or negative reaction with alkaline $AgNO_3$, but rather rapidly gave a yellow colour with periodate-Schiff's reagents. The compound, together with glycerol and a compound having the mobility and reactions of a hexitol, was formed on enzymic dephosphorylation of the mixture of organic phosphates formed on partial acid hydrolysis. Thus the former compound could be an anhydrohexitol (Baddiley, Buchanan & Carss, 1957). Insufficient material was available for more conclusive studies to be made.

Extraction of cell walls with EDTA

No specific action by EDTA was found with any of the walls from so-called resistant organisms when using conditions under which EDTA has been shown to extract a substantial proportion of the lipopolysaccharide component from the cell walls of *Pseudomonas aeruginosa* and *P. alcaligenes*. (Table 4). The absolute values for soluble phosphorus do not necessarily reflect sensitivity of the cell walls to the mildly alkaline buffer, as the walls differed in the ease with which they were deposited on centrifugation.

Table 4. *Extraction of cell-wall phosphorus by EDTA*

Cell walls were treated with 3·4 mM-EDTA in borate buffer (pH 9·2) or with buffer alone for 1 hr at 20°. Supernatant fluids were analysed after removal of insoluble residues by centrifugation.

Species	Percentage extraction of phosphorus	
	EDTA in buffer	Buffer alone
P. diminuta	9·4	9·4
P. iodinum	1·8	1·6
P. maltophilia	9·8	11·8
P. pavonacea	11·4	8·4
P. rubescens	2·3	2·1

DISCUSSION

Although several wall components have remained unidentified or have been incompletely characterized, the composition of each of the species of cell wall studied has been established in broad outline and in some detail. The bacteria selected for study were originally described as being resistant to EDTA, by using as criteria for sensitivity the release of intracellular solutes and the loss of viability under the action of EDTA. The description has now been substantiated both by the failure of EDTA to extract significant amounts of phosphorus from isolated walls and by the absence from these walls of those components which appear to characterize the lipopolysaccharides of EDTA-sensitive pseudomonads. The composition of the cell wall of *Pseudomonas iodinum* is so distinctive that it is best discussed apart from those of the other bacteria. *P. iodinum* is a non-motile, Gram-positive or Gram-variable bacterium which has been included in the genus *Pseudomonas* because it produces a phenazine pigment (Tobie, 1945; Gilman, 1953). From studies on DNA base composition and homology, its inclusion in the genus has been considered doubtful (De Ley, Park, Tijtgat & van Ermengem, 1966). Sneath (1960) suggested that the organism might belong to the genus *Corynebacterium* or to the genus *Brevibacterium*. The results of the present work confirm that *P. iodinum* is misplaced in a genus of Gram-negative organisms; the cell wall contains virtually no lipid or protein. Glycosaminopeptide components and ammonia liberated on acid hydrolysis accounted for about 95% of the total nitrogen of the cell wall. If the analytical results for amino sugars are corrected for the destruction of the pure compounds which occurred under the conditions of hydrolysis (glucosamine 32%, muramic acid 41%), the molar ratios of the glycosaminopeptide components can be calculated. The ratio glutamic acid:alanine:diaminopimelic acid:glucosamine:mura-

mic acid becomes 1·0:1·95:0·93:1·04:0·98, and the glycosaminopeptide content of the cell walls for which quantitative analyses have been given was 43%.

The material extracted from the cell wall of *Pseudomonas iodinum* by trichloroacetic acid probably is, or includes, material resembling a teichoic acid. The behaviour of the material on paper chromatography, electrophoresis and on dialysis support this view. Further evidence is provided by the detection of polyols, probably including glycerol and a hexitol, after enzymic dephosphorylation of the products obtained after partial acid hydrolysis of the material. It differed from conventional teichoic acids in not being readily precipitated from aqueous solution by acetone and in the absence of alanine. Although the amount of the material present in the cell wall was variable, it occurred in all batches of walls prepared. It is likely that galactose is a component of a separate polysaccharide also extracted from walls by trichloroacetic acid. This is suggested by the fact that the relative amounts of glucose and galactose were reversed in the batch of walls with the lower phosphorus content. This view is also supported by preliminary results on a batch of walls from *P. iodinum* organisms grown in liquid culture. The material extracted by trichloroacetic acid had an enhanced phosphorus content (8·0%) and a decreased carbohydrate content (16·6%). Glucose, but little or no galactose, was present in an acid hydrolysate of this material. No explanation has been found for the positive reaction for 2-keto-3-deoxyaldonic acids obtained with whole walls of *P. iodinum* (Table 1). However, the result is unlikely to indicate the presence of lipopolysaccharide, of which 2-keto-3-deoxyoctonic acids are characteristic components (Ellwood, 1966), as in all other respects the composition of the wall of *P. iodinum* is that expected for a Gram-positive organism.

The composition of the walls of *Pseudomonas diminuta*, *P. maltophilia*, *P. pavonacea* and *P. rubescens* is consistent with the Gram-negative reactions of these bacteria. Each species of wall contains appreciable amounts of protein and loosely bound lipid, and correspondingly decreased amounts of glycosaminopeptide. From the analyses of diaminopimelic acid and muramic acid it appears that the wall of *P. pavonacea* has the highest (about 20%) and that of *P. rubescens* the lowest (about 4%) content of glycosaminopeptides. Although the amounts of walls available were insufficient for the extraction of lipopolysaccharides, there was evidence for the presence of such compounds in each species of wall. For example, the percentage of the total phosphorus of walls extracted as loosely bound lipids ranged from 17% (*P. rubescens*) to 39% (*P. diminuta*). The situation is least clear for *P. diminuta*. Although the wall of this organism was the only one which apparently gave reactions both for a 2-keto-3-deoxyaldonic acid and for an aldoheptose, it had a very low content of phosphorus. It was also the only wall in which analysis did not show a molar excess of glucosamine over muramic acid. Both phosphorus and glucosamine are currently accepted as basal components of bacterial lipopolysaccharides. It seems unlikely that the structure of the wall of *P. diminuta* is comparable with the structures of the other walls studied.

The walls of *Pseudomonas diminuta* and *P. rubescens* had particularly high lipid contents and were notable for the presence of major amounts of glycolipids and an ornithine-containing lipid, respectively. It is possible that in *P. diminuta* the structural or functional roles of phospholipids are partly taken by glycolipids. The reactions of the ornithine-containing lipid with ninhydrin and with 2,4-dinitrofluorobenzene indicate that one amino group of the ornithine (probably that in the δ-position) is

free. The natures of the residues involved in ester and amide linkages are not yet known. However, if the amino acid is not esterified with a fatty alcohol it seems that two long-chain residues must be attached to the amino acid via the second amino group. The ornithine-containing lipid is a minor component of the cell wall of *P. maltophilia*. Phosphatidylcholine and phosphatidylinositol were not detected in any of the lipids; phosphatide composition has so far been of limited value in establishing taxonomic relationships between bacteria (Kates, 1964; Ikawa, 1967). The wall of *P. maltophilia* is distinctive for its high content of carbohydrate and for the variety of its monosaccharide components (up to 11 sugars, amino sugars and sugar acids may be present). The amount of rhamnose is so much greater than that of any other sugar that it seems likely to occur as a separate rhamnan.

Although the composition of the walls of each of the species studied is significantly different from that of *Pseudomonas aeruginosa* or *P. alcaligenes*, information about the more typical pseudomonads is not yet sufficiently extensive or detailed for taxonomic decisions to be made on the basis of the present results, except in the case of *P. iodinum*. Nevertheless, it may be said that the four other species bear no obvious relationship to the EDTA-sensitive pseudomonads nor to each other. As a result of studies on DNA it has been proposed that *P. pavonacea* and *P. rubescens* be omitted from the genus *Pseudomonas*, while *P. diminuta* was considered to be a doubtful member (De Ley *et al.* 1966). The isolated position of *P. maltophilia* relative to other aerobic pseudomonads has also been demonstrated (De Ley, 1964; Stanier, Palleroni & Doudoroff, 1966). The results of the present studies are in accord with these findings.

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Conditional Probability and the Identification of Bacteria: a Pilot Study

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SUMMARY

The use of a conditional probability model for the identification of bacteria is illustrated by a pilot study on a group consisting mainly of bacteria of the family Enterobacteriacae. It is shown that such models may be untrustworthy unless more stringent criteria are used about the basic data and about the results than is normally the case in studies of this kind, including the present one.

INTRODUCTION

In recent years the possibilities for mathematical methods in medicine have been increasingly realized. The incentive to investigate and use quantitative approaches has been greatly stimulated by the development of the electronic digital computer, since many of the calculations involved in medical problems are impractical by manual means. An early target for mathematical investigation was clinical diagnosis (e.g. Ledley & Lusted, 1959; Warner, Toronto, Veasey & Stephenson, 1961). This interest has continued, and a recent MEDLARS search produced 265 English, French and German references since 1963 under the heading of computers in diagnosis, identification and classification of disease, and bacteriology.

A common approach in clinical work has been to use Bayes' theorem or Fisher's likelihood (Kendall & Stuart, 1963*a*) to determine how seriously any given disease should be regarded as the cause of the signs and symptoms observed. For some reason, however, these methods have not been investigated to the same extent in bacteriology as in clinical medicine. An exception is the work of Möller (1962), and of Rypka, Clapper, Bowen & Babb (1967), who refer to a Bayesian programme for bacterial identification. This lack of investigation is to be regretted; bacteriology might well prove to be a more rewarding area than clinical medicine for the application of these methods, because of the reproducibility of bacteriological reactions and the opportunities for close control. Perhaps the stumbling block has been the lack of quantitative data which these theories demand; but it seemed to the present authors that even

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inspired approximations (numerically speaking) might be of some use, and the present pilot study was therefore undertaken. Although the calculations could conceivably have been carried out manually, it was felt that the greater flexibility, reliability and processing speed of a computer were needed, and a programme was written for the National-Elliott 803.

The work was begun in the latter half of 1962. A preliminary account of it was given in an article by Payne (1963). However, that account was merely expository and, as will be seen, following further investigations the present article comes to somewhat different conclusions concerning the methodology and the results.

METHODS

Bacteria studied

The bacteria chosen for study were largely members of the family Enterobacteriacae because data about this group were more readily available in a tabular form. Our data sources were Kauffmann (1954), Edwards & Ewing (1962) and *Bergey's Manual* (Breed, Murray & Smith, 1957). The list of bacteria is given in Table 1. In the study it was assumed that any unknown submitted for analysis was a strain of one of the bacteria in this list.

Tests used

The tests chosen were decided on the twin bases of available data and bacteriological experience. They are listed in Table 2.

Table 1. *List of bacteria in the reference set*

Alcaligenes faecalis	*Proteus rettgeri*	*Salmonella paratyphi* B
Alkalescens dispar group	*P. vulgaris*	*S. paratyphi* C
Arizona arizonae	*Providencia* A	*S. pullorum*
Bethesda–Ballerup group	*Providencia* B	*S. sendai*
Enterobacter aerogenae	*Pseudomonas aeruginosa*	*S. typhi*
Enterobacter cloacae	*Salmonella choleraesuis*	*S. typhimurium*
Escherichia coli	*S. choleraesuis* var. *kunzendorf*	*Shigella boydii*
E. freundii	*S. decatur*	*S. dysenteriae* 1
Hafnia alvei	*S. gallinarum*	*S. dysenteriae* 2
Klebsiella pneumoniae	*S. gallinarum* var. *duisburg*	*S. dysenteriae* (other serotypes)
K. rhinoscleromatis	*S. miami*	*S. flexneri*
Proteus mirabilis	*S. newport*	*S. sonnei*
P. morganii	*S. paratyphi* A	

Table 2. *List of tests in the reference set*

	Acid or acid+gas from
Gelatin liquefaction	Adonitol
Hydrogen sulphide production	Arabinose
Indole production	Glucose
Methyl red	Dulcitol
Motility	Inositol
Potassium cyanide (growth in)	Lactose
Sodium citrate utilization	Mannitol
Urea hydrolysis	Rhamnose
Voges-Proskauer	Salicin
	Sorbitol
	Sucrose
	Xylose

Mathematical theory

Of the two approaches considered, i.e. likelihood and Bayesian probability, it was decided not to use the Bayesian approach because of (*a*) the controversial question of the validity and role of prior probabilities in diagnosis and identification (Lipkin, 1964; Boyle *et al.* 1966); (*b*) the impossibility of estimating them in this case. These points are discussed later. We recognize, however, that both approaches are subjects for debate amongst statisticians and others, and those readers who dislike the terms 'probability' or 'likelihood' may prefer to think of the quantities concerned merely as frequency ratios.

The application of the likelihood concept to the present study runs as follows: Suppose that in past experience, out of a total of N established strains of a certain bacterium, N_1 strains had responded with a specific result, r_1 say, to a particular test, t_1. We define the *likelihood* of obtaining the specified result to the test with a further strain of the bacterium as N_1/N, i.e. the proportion of strains which in the past has given that result. This is also known as the *conditional probability* of obtaining the particular result given the test and the strain.

If with a second test, t_2 say, the proportion of the same N strains giving some other specified result r_2 is N_2/N, the likelihood that a further strain of the bacterium will give the joint pattern r_1, r_2 as results for tests t_1, t_2 is taken to be $N_1/N \times N_2/N$.

The extension to more than two tests is clear. If tests $t_1, t_2, \ldots, t_m$ were performed on N established strains of a given bacterium, and N_1 gave result r_1 for test t_1, N_2 gave result r_2 for test t_2, etc., the likelihood of obtaining the pattern $r_1, r_2, \ldots, r_m$ if the set of tests $t_1, t_2, \ldots, t_m$ is performed on a further *known* strain of the organism is taken to be

$$\frac{N_1}{N} \times \frac{N_2}{N} \cdots \frac{N_m}{N}.$$

This represents the likelihood of obtaining a specific test pattern given a *known* strain. Fisher's theory applied to the present case states that when faced with a choice of possible suspects arising from a given pattern of results with an *unknown* strain, we should make our decision by computing the likelihoods for the observed pattern for each of the possibilities, and then choose that bacterium which gives the greatest likelihood. In other words, we are to choose the suspect giving 'the greatest probability to the observed event' (Kendall & Stuart, 1963*a*). In medical work, however, a choice of alternatives often involves a comparison of the risks to the patient entailed in each alternative. For example, selection of the most likely organism in a bacteriological investigation may dictate treatment with an antibiotic noted for its unpleasant or even dangerous side effects, whereas the next most likely would require something much milder and safer. Some workers investigating the clinical applications of likelihood or Bayesian probability have attempted to allow for similar considerations by requiring that a given degree of likelihood or probability ratio between first and second suspects be exceeded before accepting that an identification has been made (e.g. Fitzgerald & Williams, 1964; Boyle *et al.* 1966).

An important assumption of the theory is that the tests must all be statistically independent. Two tests are said to be independent when the outcome of either of them is unaffected by the outcome of the other. It was assumed in the present study that the tests satisfied this assumption.

If sufficient statistics were available, then the ratios in equation (1) could be clothed in realistic numerical form. For example, if we knew that in 1000 verified strains of *Salmonella typhi*, 541 of them had failed to ferment arabinose, the ratio to use in the event of a negative result when an unknown organism was tested for fermentation of arabinose would be 541/1000 = 0·541; if the result were positive the ratio to use would be (1000–541)/1000 = 0·459. However, data such as these do not exist as yet, and it was necessary to use some relatively arbitrary values for the ratios concerned. It was decided that for tests having only two possible outcomes (or 'two-valued' tests as they were called), if a result was 'very common' or 'nearly always expected' for a particular bacterium and test, the ratio value to use would be 0·95, with the corresponding uncommon result being valued at 0·05. A more variable result was given a value of 0·5, its corresponding opposite being given the same value. Similar considerations were applied to tests having three possible outcomes ('three-valued' tests). A section of the matrix of values used is given in Table 3. The only justification we can put forward for the numbers used is that they appeared reasonable and led to fairly good results.

Table 3. *Part of the matrix of likelihoods*

		Escherichia coli	Bethesda–Ballerup	*Proteus vulgaris*	*Proteus mirabilis*	Providencia A	Providencia B
Indole	+	0·95	0·05	0·95	0·05	0·95	0·95
	−	0·05	0·95	0·05	0·95	0·05	0·05
Motility	+	0·5	0·95	0·95	0·95	0·95	0·95
	−	0·5	0·05	0·05	0·05	0·05	0·05
H_2S	+	0·05	0·95	0·95	0·95	0·05	0·05
	−	0·95	0·05	0·05	0·05	0·95	0·95
Gelatin	+	0·05	0·05	0·95	0·95	0·05	0·05
	−	0·95	0·95	0·05	0·05	0·95	0·95
Glucose	No change	0·025	0·025	0·025	0·025	0·025	0·025
	Acid	0·025	0·025	0·025	0·025	0·025	0·950
	Acid + gas	0·950	0·950	0·950	0·950	0·950	0·025
Mannitol	No change	0·025	0·025	0·950	0·950	0·950	0·475
	Acid	0·025	0·025	0·025	0·025	0·025	0·475
	Acid + gas	0·950	0·950	0·025	0·025	0·025	0·050

As an example of the use of Table 3, consider the case where tests for production of indole, for motility and for production of H_2S are applied to an unknown organism which is believed to belong to either the Providencia subgroup A or B or the *Escherichia coli* group. Let the results be +, + and − for the three tests respectively. Then, from Table 3, the likelihood of the organism belonging to Providencia subgroup A is $0{\cdot}95 \times 0{\cdot}95 \times 0{\cdot}95 = 0{\cdot}857$ (and similarly for Providencia subgroup B), and the likelihood for *Escherichia coli* is $0{\cdot}95 \times 0{\cdot}5 \times 0{\cdot}95 = 0{\cdot}451$. As far as these tests go, the maximum likelihood approach would then have us choose one of the Providencia subgroups as the most likely possibility, rather than *Escherichia coli*. Further tests would then be needed to differentiate between the two subgroups.

In the example given above, so few tests or such a ratio of likelihoods would certainly be felt to be completely inadequate for purposes of differentiation. However, the questions of how many tests *are* needed for adequate differentiation, which ones to use, and what the criterion for a decision should be are points which must be settled if

a quantitative model for identification is to be meaningful. Some attempt was made to cope with these aspects, but the problems were by no means solved, and indeed very little consideration appears to have been given to them by any other workers attempting to apply probability theory to identification in this or related areas of medicine or biology.

Percentage relative likelihood

A difficulty which arises immediately in using likelihood is the sizes of the likelihoods. Likelihood diminishes with increasing number of tests, and even with as few as 10 tests one may be dealing with quantities of the order of 10^{-3}, 10^{-4} or less. To avoid having to work with such small quantities, it was decided to express the scores arising from equation (1) above by scaling them so that the maximum for any particular set of suspects had a value of 100%. This quantity was called the *percentage relative likelihood* (see also Boyle *et al.* 1966). Thus, in the example given above, the percentage relative likelihood of each Providencia subgroup is $(0{\cdot}857/0{\cdot}857) \times 100\% = 100\%$ and that of *Escherichia coli* is $(0{\cdot}451/0{\cdot}857) \times 100\% = 52{\cdot}6\%$.

Modal likelihood fraction

At an early stage in the study it became clear that the use of likelihood or relative likelihood on its own had some serious shortcomings. Both quantities assume that probabilities or likelihoods are comparable even when they apply to different populations. Again, it has already been noted that likelihood is in general a small quantity; when using it, one is always faced with having to differentiate between a likelihood which is small because it is inherently small for the particular suspect and set of tests, and one which is small, because the suspect is unlikely or atypical on the given test results. It is not enough to say that the likelihood of A is so many times greater or smaller than that of B; we need to know also the relative status of the likelihood of A within the set of likelihoods possible for A with the given tests, and similarly for B. Relative likelihood preserves the order of the set of suspects, but it masks the difficulties of small likelihoods and likelihood distributions referred to above. To see this at work, consider an example from one of the cases tested. The test results were

Indole production	−	Lactose	No change
Motility	+	Mannitol	Acid + gas
Hydrogen sulphide	+	Rhamnose	Acid + gas
Sodium citrate utilization	+	Dulcitol	Acid + gas
Gelatin hydrolysis	−	Arabinose	Acid + gas
Potassium cyanide (growth in)	−		
Xylose	Acid + gas	Sorbitol	Acid + gas
Sucrose	No change	Inositol	No change

with results

Possible organism	% Relative likelihood
Salmonella newport	100·00
S. paratyphi C	50·00
S. paratyphi B	6·58
S. typhimurium	6·58
S. choleraesuis var. *kunzendorf*	1·32

On these results, *Salmonella newport* is twice as likely as *S. paratyphi C* (the correct answer) and nearly 16 times as likely as *S. paratyphi B* or *S. typhimurium*. But on further examination of the matrix of values, it became clear that the absolute likelihoods for the first four organisms given above were the *greatest possible with the given tests.*

Moreover, the procedure for selecting further tests (see below) showed that none of the remaining tests had any power to increase the discrimination. In other words, because *S. paratyphi C* and the others are more variable in their behaviour than *S. newport* as far as the given tests are concerned, they were being discriminated against because of their variability. This is clearly unrealistic. Other implications of this situation will be raised in the Discussion. Theory is available which could perhaps cope with this situation to some extent (e.g. Kendall & Stuart 1963*b*), but at the time of this study the authors were (and still are) unaware of any theory which would deal completely with the mixture of two-valued and three-valued tests being used. An empirical approach was therefore used which, nevertheless, has some statistical justification, as will be shown.

This approach was to compute what was called the *modal likelihood fraction* for each suspect. This was defined as

$$\frac{\text{likelihood for the given set of test results}}{\text{maximum likelihood possible for that bacterium with the given tests}}.$$

Thus in the previous example, the modal likelihood fraction for each of *Salmonella newport, S. paratyphi C, S. paratyphi B* and *S. typhimurium* was found to be 1·0. This showed that the likelihoods for each were the best possible with the given tests, and implied that it would be misleading to choose *S. newport* in preference to *S. paratyphi C* or even *S. typhimurium*, on the likelihoods given. Further tests were undoubtedly needed.

If we regard the ratios N_1/N in (1) as probabilities, the modal likelihood fraction expresses the ordinate, corresponding to the actual test results in the multivariate distribution associated with a given bacterium for a given set of tests, as a fraction of the *mode* of the distribution, hence the name. For a 'well-defined' organism, i.e. sharply differentiable over a set of tests, the distribution should be unimodal and the bulk of it should lie around the mode; the more evenly the distribution lies, the more diffuse the organism. On the assumption of reasonably well-defined organisms, the modal likelihood fraction is an attempt to compare the rarity of patterns within their distributions.

Selection of further tests

As well as using the matrix of ratios for the selection of possibilities, an attempt was made to use it for the selection of further tests aimed at improving the separation between suspects. Later work has shown that the method does not necessarily lead to an improvement in relative likelihood separation between suspects. However, a brief account of the approach is worthwhile in that it bears some resemblance to the method recently described by Rypka *et al.* (1967) and it did prove possible to use it to discover the point at which further testing could lead to no improvement at all in separation.

Consider in Table 3 the example of the reactions for gelatin liquefaction and H_2S production of the Bethesda–Ballerup group, *Proteus vulgaris* and *P. mirabilis*. We see that the gelatin liquefaction test cannot distinguish between the two Proteus species: the distribution of values with respect to positive and negative outcomes is exactly the same for both organisms, and, whatever the test result, we would gain no additional discrimination. However, testing for gelatin liquefaction is clearly of use in distinguishing the Bethesda group from the two Proteus species; a common result for one is uncommon for the other, and vice versa. On the same lines, the test for H_2S production

is useless for distinguishing between any of the organisms. The reasoning is the same with three-valued tests: testing for fermentation of glucose adds nothing to the discrimination between the three organisms, whereas the mannitol fermentation test can help to some extent.

This reasoning led to consideration of *the absolute difference between corresponding ratios* as a measure of discriminating power. The power of a test to distinguish between two bacteria was defined as *the sum of the absolute differences between corresponding ratios for all possible outcomes to the test.* Thus, for example, the power of the gelatin liquefaction test to distinguish between the Bethesda group and *Proteus vulgaris* would be:

$$|0{\cdot}05-0{\cdot}95|+|0{\cdot}95-0{\cdot}05| = 1{\cdot}8,$$

and that of mannitol fermentation:

$$|0{\cdot}025-0{\cdot}950|+|0{\cdot}025-0{\cdot}025|+|0{\cdot}950-0{\cdot}025| = 1{\cdot}85$$

(where we use the normal mathematical notation for an absolute value: $|x| = x$ if x is positive, $|x| = -x$ if x is negative). This would imply that mannitol fermentation is slightly better than gelatin liquefaction in this case.

For a set of bacteria, the power of a test to distinguish between them was obtained by making the calculation for all possible pairs in the set and summing the results. This consideration of all possible pairs of differences is comparable with the procedure of Rypka *et al.* (1967) and would lead to similar results in the case of non-variable characters. It also enlarges their method to multiple-valued tests. However, the variability of the characters led to a more complex situation than that envisaged by Rypka *et al.* and in fact it can be shown that with two-valued tests the procedure cannot be relied on to lead to an improvement in relative likelihood separation. We have been unable so far to demonstrate an analogous result for three-valued tests, but it is almost certain that the situation is the same for these also. The 'Discriminating Power', as it was called, had some usefulness; it gave a measure of the spread of the distribution of ratios for a test over a set of bacteria and was able to indicate when a test could not discriminate at all over a set of suspects. It also gave more power to a 'good' three-valued test than to a 'good' two-valued one, corresponding with what one would expect on heuristic grounds. However, apart from using it to decide when tests were useless, the method was not investigated to any depth and it is not proposed to discuss it further, though the Discussion shows that some method is necessary for determining the sequence in which tests are carried out.

Short description of programme and procedure

The programme was designed to take in first as data the matrix of values; each row was sum-checked to guard against reading and data-punching errors. Once the matrix had been read in, the details of the individual case followed. The first of these was a number to indicate whether the case was a new one or a continuation of a previous calculation. If a continuation, the programme expected the data output at the end of the previous run for this case before it would read in the new data, otherwise the programme went straight into reading in the new data. The new data consisted of a set of test numbers (each test was given a number between 0 and 20 inclusive), each test number being followed by a result number. With two-valued tests, this was zero for + and 1 for −; with three-valued ones, 'no change' was coded zero, 'acid'

coded 1, and 'acid+gas' coded 2. After the test/result statements came parameters to determine how many suspects to print out, and how many further tests to select. The programme then did its calculations and printed its answers. Then, according to the user's decision on studying the answers, it could either return to take in results of further tests for the same case or data for an entirely new case. There were various refinements built in—e.g. a case was automatically ended when only one bacterium was printed out or when there were no further tests with any discriminating power. The calculation for any given set of data took 20 to 40 sec., depending on the number of tests and the values of the various parameters.

The intention in this study was not so much to achieve identifications as to investigate the outcome of taking the 100% relative likelihood suspect to be the correct answer at various stages of testing. This is the straight maximum likelihood approach; as already noted, other workers have required arbitrary levels of likelihood ratio between first and second suspects to be exceeded before accepting the most likely suspect as the answer. There was therefore no particular criterion used for terminating specific investigations except when either the odds of first choice to second choice became so high that it appeared extremely unlikely that further tests would make any difference, or else the procedure for selection of further tests showed that no further discrimination was possible.

RESULTS

Cases investigated

Apart from several test cases used during the programme development, 12 cases were investigated. These consisted of a known specimen of each of: *Salmonella typhimurium*, *Shigella dysenteriae* (not serotype 1 or 2), *Salmonella paratyphi A*, *S. paratyphi B* and *S. paratyphi C*, *Arizona arizonae*, *Escherichia coli*, *Shigella boydii*, *S. sonnei*, *Klebsiella pneumoniae*, *Providencia* subgroup A and *Proteus mirabilis*. In each case an independent Reference Laboratory determination was available for comparison with the computed result. An example of the process (which also illustrates the output from the programme) is given in the Appendix. Table 4 gives a summary of results for the 12 cases.

DISCUSSION

Even with so few cases, the examples already discussed and the results given in Table 4 reveal some undesirable features of the use of likelihood for this type of work. Further points (see below) indicate that methodological deficiencies can become extremely serious. It is worth noting that Bayes' theorem shares these deficiencies, over and above the difficulties arising out of the use of prior probabilities. The first point has already been indicated, namely that when using likelihood alone, the more variable organisms are discriminated against, because of their variability. What this amounts to is that a given set of tests may have insufficient discriminating power to effect a conclusive separation between the organisms it is supposed to cover, even under the most favourable circumstances. The operative words are 'insufficient' and 'conclusive', because the user has at least two options open to him: (1) to decide in advance what will be an acceptable likelihood ratio between first and second suspects for a decision and then check (by means of modal likelihood fraction or some other method) that the set of tests can meet this norm under the most favourable circumstances or under some defined deviation from them; (2) to accept the test set as it

stands, and determine the worst misclassification which can occur, again under some defined set of circumstances, and then use the likelihood ratio level for which this occurs as the level to be exceeded before a decision can be made. Thus, if on the *Salmonella* example given earlier, this strain had contributed to the basic table of likelihoods and the true answer was known to be *Salmonella typhimurium*, and it was also known that the ratio of relative likelihoods of 100:6·58 was the highest for any misclassification under optimum circumstances, then the criterion would be that the ratio between first and second suspects must be at least 100:6·58 before a decision could be made.

Whatever procedure is adopted, the blind use of a set of tests combined with either simple maximum likelihood choice or an arbitrary discrimination level between likelihoods is untenable and inconsistent. Unfortunately this appears to have been the standard procedure in all the published work known to the authors on the use of conditional probability for diagnosis and identification, not just in bacteriology but in medicine generally. Criteria for discrimination are not based on a critical evaluation of the basic data, as they should be.

A second criticism which can be made is that results depend on the order in which tests are done. For instance, in case 5 (Table 4) the correct organism started off after five tests as the 'most likely' (though it was in fact one of a set of seven suspects all with 100% relative likelihood); after two more tests it had a relative likelihood of 50%, becoming 100% again after eight more tests. Had the tests been made in a different order with different groupings, the sequence of percentages would have been different: the correct organism would have remained at 100% throughout. It is interesting to see from Table 4 that the complete set of tests was in this case shown to be inadequate for an unequivocal separation on the given results. In this case, even if one were prepared to accept a 2:1 ratio as a basis for decision, confirmatory tests would probably have been desirable after the 50% relative likelihood level had been reached, and a mistaken identification would have been avoided.

But whereas in this example the effect of the sequence was unimportant, in other examples it was more serious, and this brings the third criticism, the possible vulnerability of the procedure to errors in data. In case 2 (Table 4), the correct result started off with a relative likelihood of less than 10% after five tests; the level rose to 21% after six more tests and remained at this level through four confirmatory tests. Only with the final three tests did the identification become dubious with a rise to 84%. Had one been discriminating at a ratio of 5:1 (not an unreasonable level, perhaps), it would have been tempting to consider the identification confirmed after the fourth set of tests. The point about this example is that one of the test results was found later to have been erroneously coded. The result for sucrose fermentation was coded as 'acid + gas', whereas the true result was 'no change', and the systematic depression of the results for *Salmonella paratyphi A* was due entirely to this one error. With the correct result for the test, the outcome after the second group of tests would have been 100:25 in favour of *S. paratyphi A* against *Escherichia coli*, with a modal likelihood fraction of 0·013 for the former; this ratio would have persisted until the fifth group of tests, when the final ratio between these two would have been 100:6. The original results favoured *E. coli*, and, what is more, had these results been taken in a different order, the ratio of 100:84 would have been arrived at earlier in the sequence, giving a final 'confirmed' ratio of 100:84 for *Escherichia coli* against *Salmonella paratyphi A*. Another case (number 9) had an unusual result (urea hydrolysis negative)

Table 4. *Summary of twelve cases*

The numbers in the columns marked 'Test set' indicate whether the test concerned was among the first group of tests used, or the second, or the third etc. The numbers in the columns marked '% r.l.' and 'm.l.f.'—corresponding to 'percentage relative likelihood' and 'model likelihood fraction' respectively—indicate the values of these quantities for the correct suspect after the corresponding test set had been completed. Thus, for Case 1, alleged to be *Shigella boydii*, the percentage relative likelihood for *S. boydii* at the completion of the second group of tests was 13·2 %, and the modal likelihood fraction was 1·00.

Case no. ...	1 *Shigella boydii*			2 *Salmonella paratyphi A*			3 *S. typhimurium*			4 *S. paratyphi C*			5 *S. paratyphi B*			6 *Shigella dysenteriae* (other)		
Tests	Test set	% r.l.	m.l.f.	Test set	% r.l.	m.l.f.	Test set	% r.l.	m.l.f.	Test set	% r.l.	m.l.f.	Test set	% r.l.	m.l.f.	Test set	% r.l.	m.l.f.
Gelatin hydrolysis	.	.	.	4	.	.	3	.	.	3	.	.	3	.	.	6	100·0	0·026
H_2S produced	1	.	.	1	.	.	1	.	.	1	.	.	1	.	.	1	.	.
Indole produced	1	.	.	1	.	.	1	.	.	1	.	.	1	.	.	1	.	.
Methyl red	.	.	.	2	.	.	.	.	.	.	.	.	.	.	.	3	.	.
Motility	1	.	.	1	.	.	1	.	.	1	.	.	1	.	.	1	.	.
KCN growth	.	.	.	4	.	.	3	.	.	3	.	.	3	.	.	.	.	.
Sodium citrate	3	100·0	1·00	3	21·0	0·0007	3	.	.	3	.	.	3	.	.	.	.	.
Urea hydrolysis	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	.	.
Voges–Proskauer	.	.	.	4	21·0	0·0007	.	.	.	.	.	.	.	.	.	.	.	.
Adonitol	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Arabinose	2	.	.	2	.	.	2	.	.	2	.	.	2	.	.	3	100·0	0·026
Glucose	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	4	100·0	0·026
Dulcitol	2	.	.	5	.	.	2	100·0	1·00	2	50·0	1·00	2	50·0	1·00	5	100·0	0·026
Inositol	.	.	.	5	.	.	3	.	.	3	.	.	3	.	.	.	.	.
Lactose	1	.	.	1	.	.	1	.	.	1	.	.	1	.	.	1	.	.
Mannitol	2	.	.	2	.	.	3	.	.	3	.	.	3	.	.	2	.	.
Rhamnose	.	.	.	2	.	.	3	.	.	3	.	.	3	.	.	.	.	.
Salicin	.	.	.	*5	**84·2**†	**0·0007**	.	.	.	.	.	.	.	.	.	.	.	.
Sorbitol	.	.	.	2	.	.	3	.	.	3	.	.	3	.	.	.	.	.
Sucrose	1	52·7	1·00	1	< 10	.	1	100·0	1·00	1	100·0	1·00	1	100·0	1·00	1	100·0	0·026
Xylose	2	13·2	1·00	2	21·0	0·0007	*3	**100·0**	**1·00**	*3	**50·0**	**1·00**	*3	**100·0**	**1·00**	2	100·0	0·026
Total no. of tests per case	11			18			15			15			15			13		

Table 4 (*cont.*)

Case no. ...	7 *Arizona arizonae*			8 *Proteus mirabilis*			9 *Klebsiella pneumoniae*			10 Providencia subgroup A			11 *Escherichia coli*			12 *Shigella sonnei*		
Tests	Test set	% r.l.	m.l.f.	Test set	% r.l.	m.l.f.	Test set	% r.l.	m.l.f.	Test set	% r.l.	m.l.f.	Test set	% r.l.	m.l.f.	Test set	% r.l.	m.l.f.
Gelatin hydrolysis	4	.	.	3	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H_2S produced	1	.	.	1	.	.	1	.	.	1	.	.	1	.	.	1	.	.
Indole produced	1	.	.	1	.	.	1	.	.	1	.	.	1	.	.	1	.	.
Methyl red	.	.	.	.	.	.	2	.	.	.	.	.	.	.	.	.	.	.
Motility	1	.	.	1	.	.	1	.	.	1	.	.	1	.	.	1	.	.
KCN growth	3	.	.	3	.	.	.	.	.	.	.	.	3	.	.	2	.	.
Sodium citrate	.	.	.	2	.	.	.	.	.	.	.	.	3	100·0	1·00	.	.	.
Urea hydrolysis	1	.	.	1	.	.	1	.	.	1	.	.	1	.	.	1	.	.
Voges–Proskauer	.	.	.	.	.	.	2	1·0	0·003	.	.	.	.	.	.	.	.	.
Adonitol	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Arabinose	2	.	.	.	.	.	.	.	.	2	.	.	.	.	.	.	.	.
Glucose	3	.	.	3	.	.	.	.	.	3	100·0	0·026	2	.	.	.	.	.
Dulcitol	2	50·0	0·026	2	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Inositol	4	**100·0**	0·0007	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Lactose	1	.	.	1	.	.	1	.	.	1	.	.	1	.	.	1	.	.
Mannitol	3	.	.	3	100·0	0·053	.	.	.	2	100·0	0·026	2	.	.	3	100·0	1·00
Rhamnose	3	100·0	0·026	2	.	.	.	.	.	.	.	.	2	.	.	.	.	.
Salicin	.	.	.	.	.	.	3	**2·0**	**0·003**	.	.	.	.	.	.	2	.	.
Sorbitol	.	.	.	2	100·0	0·053	.	.	.	.	.	.	2	100·0	1·00	.	.	.
Sucrose	1	100·0	1·00	1	100·0	1·00	1	1·0	0·003	1	100·0	0·026	1	100·0	1·00	1	100·0	1·00
Xylose	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	2	100·0	1·00
Total no. of tests per case	14			14			9			9			12			10		

* No further tests available with discriminating power between the suspects printed.
† Bold final scores mean that at least one other suspect had the same score or greater.

as well as an erroneous one (motile strain). The effect of the correct coding would have been to leave the relative position of the correct answer unchanged, but its relative likelihood would have risen to 20%, and its modal likelihood fraction to 0·052.

The two previous criticisms make it clear that the practical use of this technique requires rigorous data-checking, and also requires a criterion for deciding the sequence in which tests are to be carried out. Such a sequence will of necessity be dynamic, in that fresh tests will need to be determined at any stage on the basis of results up to that stage. To what extent the methods of Rypka *et al.* (1967) already referred to, or those of Gyllenberg (1964), can be used has yet to be seen, but it is possible that the lack of resolution of these methods where variable characters are concerned may render them unsuitable. A more promising approach may be to evaluate at each stage the increase either in probability difference or in probability ratio between first and second suspects for all possible outcomes of all remaining tests and then to choose the test(s) giving the greatest minimum increase—in other words, choose those tests whose most unfavourable outcome is better than that of the other tests. Whatever technique is adopted should be tested by growing strains, dividing the cultures and investigating one half by orthodox laboratory methods and the other by following the computed sequence. Only by such practical investigations can a theoretical method prove its worth.

A final criticism which can be levelled is that the procedure as given (and this is true of any study using either unaided Bayesian probability or (relative) likelihood) is incapable of recognizing a strain from outside its basic reference set. Any such strain will be identified as the member of the reference set which most resembles it, even if the resemblance is derisory.

As against the criticisms, it is true that the maximum relative likelihood choice would have had some success. Six of the twelve specimens would have been unequivocally identified, and a further three would have been among the choices with 100% relative likelihood. One case would have been identified unequivocally if it had not been for a data error, one was incapable of being identified on the test results, and one case would have been misidentified even if an erroneous test result had not been included. But a higher success rate is needed if the procedure is to be practical. Bearing in mind the points previously made, one is left with the uncomfortable feeling that good luck played its part as much as good judgement. Perhaps more realistic frequency ratios might have improved the performance in the marginal cases. But the acquisition of such data would call for a national or international effort, as has been noted in another context (Bogdanescu & Racotta, 1967).

Conclusions

All in all, we submit that the present study, limited as it was, shows that the use of conditional probability for bacteriological identification is unlikely to prove satisfactory unless the basic data are critically evaluated beforehand and further investigations are made of the techniques of decision-making based on conditional probability. In addition, a rationale for the determination of test sequences is also needed.

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APPENDIX

Example of program output; strain, Providencia subgroup A

First set of tests

Given	Identification
Indol	+
Motility	+
H_2S	−
Urea	−
Sucrose	Acid + gas
Lactose	Acid

Then, applying these results to the complete set of bacteria, the most likely are (taking either the first 10 or those whose % relative likelihood is greater than 1 %, whichever is less):

	Relative likelihood (%)	Modal likelihood fraction
Providence gp. (Biochem gp. 1)	100.000000	0·026316
E. coli group	52·631579	0·105263
Alk./dispar group	5·263158	0·005540
Cloaca cloaca	2·915877	0·005540
Providence gp. (Biochem. gp. 2)	2·631579	0·000693

The best tests for distinguishing between these possibilities are (taking either the first six or those whose power is greater than 0·80 of the maximum power scored whichever is less):

	Relative discriminating power
Arabinose	1·000
Mannitol	0·9966

First and second set of tests

Given	Identification
Indole	+
Motility	+
H_2S	−
Urea	−
Sucrose	Acid + gas
Lactose	Acid
Mannitol	No change
Arabinose	No change

Then, applying these results to the complete set of bacteria, the most likely are (taking either the first 10 or those whose % relative likelihood is greater than 1 %, whichever is less):

	Relative likelihood (%)	Modal likelihood fraction
Providence gp. (Biochem. gp. 1)	100·000000	0·026316
Providence gp. (Biochem. gp. 2)	1·315789	0·000693

The best tests for distinguishing between these possibilities are (taking either the first six or those whose power is greater than 0·80 of the maximum power scored, whichever is less):

	Relative discriminating power
Glucose:	1·00000

First, second and third set of tests

Given	Identification
Indole	+
Motility	+
H_2S	−
Urea	−
Glucose	Acid + gas
Sucrose	Acid + gas
Lactose	Acid
Mannitol	No change
Arabinose	No change

Then, applying these results to the complete set of bacteria, the most likely are (taking either the first eight or those whose % relative likelihood is greater than 1 %, whichever is less):

	Relative likelihood (%)	Modal likelihood fraction
Providence gp. (Biochem. gp. 1) (only one bacterium)	100·000000	0·026316

Notes

(*a*) *The names of tests and organisms used in the output were working names, and no attempt was made to ensure that these conformed to internationally accepted nomenclature. The titles 'Providence gp. (Biochem. gp. 1)' and 'Providence gp. (Biochem. gp. 2)' refer to Providencia subgroup A and Providencia subgroup B, respectively. The other titles will be self-evident.*

(*b*) *In the programme, the 'discriminating power' for any test over a set of bacteria was scaled by the maximum for the tests being considered, and referred to as 'relative discriminating power'.*

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The Control Mechanism of Sclerotial Formation in *Sclerotium rolfsii* Sacc.

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SUMMARY

The distribution of [^{14}C]-L-cysteine and [^{14}C]iodoacetic acid in mycelium and sclerotia of *Sclerotium rolfsii* Sacc., and the effect of disodium ethylenediaminetetraacetic acid (Na_2EDTA), trans-1,2-diaminocyclohexane-*N*,*N*,-*N*′,*N*′-tetraacetic acid (Chel. C.D.), potassium iodate and phenylthiourea on the formation of sclerotia by *S. rolfsii* were studied. [^{14}C]-labelled iodoacetic acid accumulated specifically in the sclerotia, whereas [^{14}C]-L-cysteine was equally distributed throughout the mycelium. Accumulation of iodoacetic acid at specific sites was observed even before the formation of the sclerotia. Most of the radioactivity of the fungal mycelium grown on [^{14}C]iodoacetic acid was found in the cell-free extract, 93% of the radioactivity of the extract being associated with the ammonium sulphate-precipitated fraction. Na_2-EDTA, Chel. C.D. and potassium iodate at 10^{-3}M also induced sclerotial formation. The effect of Na_2EDTA was eliminated by the addition of 3×10^{-5}M-Cu^{2+}, but not by Fe^{2+}, Zn^{2+}, Co^{2+}, Mn^{2+}, Ca^{2+} nor Mg^{2+}. Phenylthiourea (10^{-3}M) initiated sclerotial formation but inhibited further development and melanogenesis. It is suggested that sclerotial formation in *S. rolfsii* is induced by inactivation of a $-SH+Cu^{2+}$-containing protein entity which acts as a repressor of sclerotial formation.

INTRODUCTION

It was reported by Chet, Henis & Mitchell (1966) that sclerotial production in *Sclerotium rolfsii* could be either prevented by L-cysteine or induced by iodoacetic acid, the compounds completely neutralizing each other at a molar ratio of 30:1. The high ratio required for this annulment might have resulted from differential cell permeability towards the compounds, or from differences in their distribution inside the hyphal cells. The site of action and target of these compounds might have been cell structures in which –SH groups are of significance, including the cell wall (Nickerson, 1963), or enzymes involved in morphogenetic processes (Brachet, 1964). Any working hypothesis which explains the effect of sulphur-containing amino acids and their antagonists on sclerotial formation in *S. rolfsii* must also include their distribution and binding sites inside the fungal cell as well as mechanical factors involved in sclerotial formation by *S. rolfsii* (Henis, Chet & Avizohar-Hershenzon, 1965). Experiments made to investigate these problems, and a working hypothesis to explain the control mechanism of sclerotial formation in *S. rolfsii*, are presented in the present paper.

METHODS

Organism. The *Sclerotium rolfsii* strain used in these experiments was isolated from sugar beet.

Radioautograms. Sclerotium rolfsii was grown on Petri dishes containing 15 ml. defined medium agar (Joham, 1943), supplemented with 5 μc. of either 2-[^{14}C]iodoacetic acid (specific activity 35·0 μc./mg) or 3-[^{14}C]-L-cysteine (specific activity 32·0 μc./mg) obtained from The Radiochemical Centre, Amersham, Buckinghamshire, U.K. The final concentration of these compounds was adjusted to 10^{-4}M and 10^{-2}M, respectively. Colonized agar discs, 0·5–1·5 cm. in diameter, were used as inocula. The cultures were incubated for 15 days at 30°.

The agar medium was separated from the fungal colonies by melting the agar. The mycelial mats were washed with hot distilled water and dried in air on a glass cover of a Petri dish. A Kodak Medical X-ray film was then pressed firmly against the mycelium, and the whole placed in the dark for 5 days.

Distribution of radioactivity. Sclerotium rolfsii was grown in Petri dishes containing 15 ml. liquid defined medium supplemented with 5 μc. labelled iodoacetic acid or L-cysteine adjusted to 10^{-4}M and 10^{-2}M, respectively, inoculated and incubated as described. Quantitative estimations of radioactivity were done with a Packard Tricarb Scintillation spectrometer model 3365, using dried, thinly ground powder of either cell walls (Chet, Henis & Mitchell, 1967), mycelium or sclerotia. Cell-free extracts were tested for radioactivity in a liquid form. Counts were calculated on a weight basis (dpm/mg. dry weight).

Preparation of cell-free extract. Sclerotium rolfsii was grown in a shallow layer of liquid defined medium (Joham, 1943) for 10 days at 30°. Twenty g. (wet weight) of washed mycelium were taken in 20 ml. phosphate buffer (M/40; pH 6·2), broken in a Waring Blendor for 10 min. and treated in an MSE ultrasonic disintegrator for 5 min. The remaining cells and cell debris were precipitated by centrifugation (12,000 *g* for 10 min.) at 2°.

Protein determination. Protein content of the cell-free extract was determined by the Folin phenol method of Lowry, Rosebrough, Farr & Randall (1951).

Assay of cysteine reductase activity of the cell-free extract. This was done by following the oxidation of $NaDH_2$ (A grade, Calbiochem, Lucerne, Switzerland) in a Shimadzu UV spectrophotometer, at 340 mμ (Nickerson & Romano, 1952).

RESULTS

Distribution of radioactivity in Sclerotium rolfsii grown on labelled iodoacetic acid or L-cysteine

Quantitative determinations of the radioactivity of various parts and fractions of *Sclerotium rolfsii* confirmed the results obtained with the radioautography technique (Table 1). Whereas radioactivity of sclerotia and mycelium of *S. rolfsii* grown on [^{14}C]-L-cysteine differed only slightly, radioactivity in colonies grown on [^{14}C]iodoacetic acid accumulated specifically in the sclerotia. Radioautograms of colonies grown on agar medium supplemented with [14C]-labelled iodoacetic acid showed a specific accumulation of radioactivity in the fungal sclerotia (Pl. 1, fig. 1, 2). Accumulation of radioactivity at specific sites was observed before any sclerotia were formed (Pl. 1,

fig. 3, 4). The distribution of these sites on the fungal colony was very similar to that of sclerotia formed on an iodoacetate-supplemented medium. On the other hand, when the fungus was grown on media containing 10^{-2}M-L-cysteine, radioactivity was distributed equally throughout the mycelium (Pl. 1, fig. 5, 6). When cell-free extract of fungal mycelium grown on [^{14}C]iodoacetic acid was fully saturated with ammonium sulphate and centrifuged for 10 min. at 12,000 *g*, 93% of its radioactivity was found in the precipitate.

Table 1. *Distribution of radioactivity in mycelium and sclerotia of Sclerotium rolfsii grown in* [^{14}*C*]-L-*cysteine or* [^{14}*C*]*iodoacetic acid*

Compound added to growth medium	Tested form or fraction	dpm/mg.* dry weight	Standard error of the mean
Iodoacetic acid 10^{-4} M	Sclerotia	365	7·2
	Mycelium	153	6·1
L-cysteine 10^{-3} M	Sclerotia	216	6·1
	Mycelium	182	7·1
Iodoacetic acid 10^{-4} M	Cell-free extract	359	5·8
	Hyphal cell walls	119	5·1

* Each value represents the mean of four replicates.

Effect of chelating agents on sclerotial formation

The possibility was considered that sulphur-containing amino acids might have affected sclerotial formation through a chelating mechanism (Jones, 1964; MacLeod, Smith & Gelinas, 1966). If so, other chelating agents might have a similar effect on sclerotium formation. Disodium ethylenediaminetetraacetic acid (Na_2EDTA) and trans-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid (Chel. C.D.) obtained from Fluka A.G., Switzerland, were added to the defined medium agar at final concentrations of 10^{-4} to 10^{-2}M. The agar plates were inoculated with *Sclerotium rolfsii* and incubated for 10 days at 30°. The results are shown in Pl. 2, fig. 1, 2. As with iodoacetic acid, sclerotia were formed in concentric circles around the inoculum discs. However, they were unusually big, soft and light yellow in colour. They also fused into each other and formed irregularly shaped masses. Upon the addition of Cu^{2+} ions at $3{\cdot}10^{-5}$ M to 10^{-2} M-Na_2EDTA-supplemented medium, sclerotial formation and appearance resembled the unsupplemented control (Pl. 2, fig. 3). Other ions (Fe^{2+}, Mn^{2+}, Zn^{2+}, Mg^{2+}, Co^{2+} and Ca^{2+} at 10^{-4}–10^{-2}M) did not neutralize the effect of Na_2EDTA.

Effect of phenylthiourea and potassium iodate on sclerotial formation

Potassium iodate at 10^{-3}M induced sclerotial formation in a way similar to that of iodoacetic acid, whereas phenylthiourea, a potent inhibitor of melanogenesis (Whittaker, 1966), induced the formation of sclerotial initials, but prevented their further development and maturation. However, colony growth was also strongly inhibited (Pl. 2, fig. 4).

'Cystine reductase' activity in Sclerotium rolfsii

The presence of this enzymic activity of *S. rolfsii* is evident from Fig. 1.

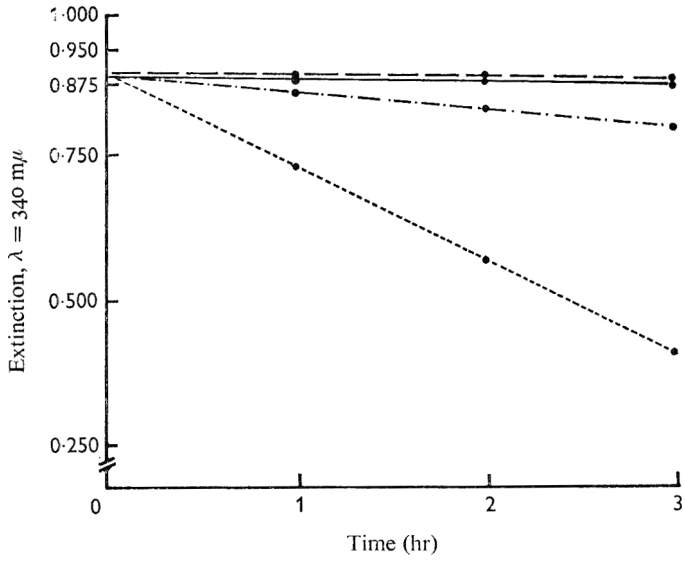

Fig. 1. Cystine reductase activity of cell-free extract of *Sclerotium rolfsii*. Reaction mixture contained 1 ml. cell-free extract (500 μg. protein/ml.), 12 ml. phosphate buffer (M/40, pH 6·2). 1 ml. buffer solution saturated with cystine (0·112 mg./ml.). and 300 μg. $NADH_2$. Boiled control was heated for 5 min. at 100°. Reaction mixture was incubated in a Dubnoff shaking bath at 37°. Readings were made in a Shimadzu UV spectrophotometer at 340 mμ. – – –, boiled extract + buffer (pH 6·2); –·–·–, $NADH_2$ + extract + buffer; ——, $NADH_2$ + cystine + buffer; - - - - -, $NADH_2$ + cystine + extract + buffer, in a total volume of 14 ml.

DISCUSSION

Radioautograms of *Sclerotium rolfsii* grown on [^{14}C]iodoacetic acid showed a selective accumulation of radioactivity in the sclerotia. An increase in radioactivity at specific sites was observed in fungal colonies grown on iodoacetic acid before the initiation of sclerotial formation, these sites being distributed on the fungal colonies in a way similar to that of sclerotia on iodoacetate-grown colonies. In contrast to iodoacetic acid-grown cultures, radioactivity of colonies grown on [^{14}C]-L-cysteine was equally distributed throughout the mycelium and in the few sclerotia which were formed. Thus, the relatively high concentrations of L-cysteine required to neutralize the effect of iodoacetic acid on *S. rolfsii* (Chet *et al.* 1966) may be partially explained by their different distribution in the fungal mycelium.

Na_2EDTA and potassium iodate induced sclerotial formation in concentric circles around the inoculum and induction by Na_2EDTA was completely prevented only by Cu^{2+}. However, induction of sclerotial formation by potassium iodate may indicate that oxidation processes are also involved.

Felix & Brouillet (1966) isolated –SH-containing peptidases from brewer's yeast; both enzymes were inhibited by *p*-chloromercuribenzoate and iodoacetate. However, only in one enzyme was the inhibition partially prevented by L-cysteine. This enzyme was not affected by EDTA. The other was EDTA-sensitive, Ca^{2+} and Zn^{2+} being effective in preventing the inhibitory effect of EDTA. Schramm (1964) showed the presence of masked –SH groups and calcium ions in pancreatic α-amylase. Under normal conditions, amylase was not affected by iodoacetate, iodoacetamide and

dithio-bis-2-nitrobenzoic acid (DTNB). Upon adding EDTA, some –SH groups became unmasked and reacted with DTNB and with *N*-methylimide, suggesting that masking of sulphydryl groups was mainly due to the tightening of the enzyme structure by bound calcium. On the basis of these observations, it is tempting to suggest that iodoacetic acid, chelating agents and potassium iodate induce sclerotial formation in *Sclerotium rolfsii* by modifying a sulphydryl-containing copper-linked protein entity which acts as a repressor of sclerotial formation; L-cysteine may decrease sclerotial formation by increasing its activity or its formation, or by slowing the rate of its turnover. Cystine can replace L-cysteine, being converted to the latter by 'cystine reductase' activity (Nickerson & Romano, 1952) found in the mycelium of *S. rolfsii*. The formation of sclerotia in concentric circles, induced by iodoacetic acid, potassium iodate or Na_2EDTA, might have resulted from their specific accumulation, up to a critical concentration, at the growing hyphal tips of the fungal colony, causing the local inactivation of this hypothetical $-SH + Cu^{2+}$-containing repressor. The induction of sclerotial formation by mechanical means such as tearing or cutting (Henis *et al.* 1965) may also be explained by the inactivation of this repressor either by oxidation of –SH groups or by the accumulation of internal natural chelates such as organic acids as a result of nutrient flow to the damaged area. This explanation may also hold for the tendency of the fungus to produce sclerotia upon creating a contact with the side wall of the Petri dish. Such a contact temporarily arrests growth, and may result in the local increase of nutrients, flowing from the centre of the fungal colony toward its hyphal tips at the growing edge. It is believed that this working hypothesis will serve as a useful tool in further studies on the morphogenesis of *S. rolfsii*.

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EXPLANATION OF PLATES

PLATE 1

Photographs (left) and corresponding radioautograms (right) of mycelial mats of *Sclerotium rolfsii* grown on a defined medium agar supplemented with [^{14}C]-L-cysteine or [^{14}C]iodoacetic acid.

Figs. 1, 2. [^{14}C]iodoacetic acid (10^{-4} M). Photographs taken after 15 days of incubation at 30°. (× 1.)

Figs. 3, 4. As in fig. 1 and 2, after 24 hr. (× 4.)

Figs. 5, 6. ^{14}C-L-cysteine (10^{-2} M). Photographs taken after 15 days of incubation at 30°. (× 1.)

PLATE 2

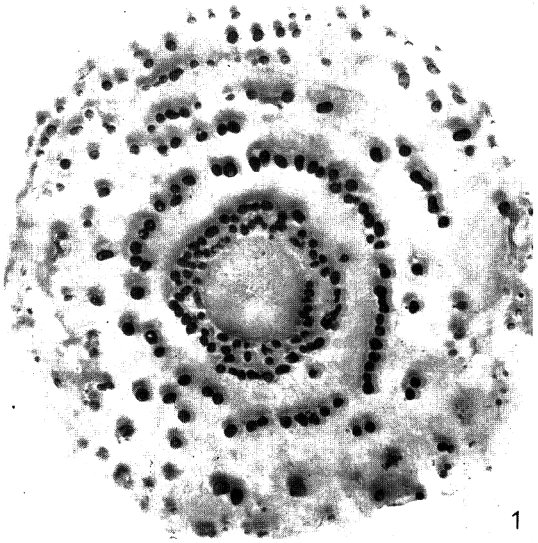
Effect of chelating agents and phenylthiourea on sclerotia formation in *Sclerotium rolfsii*, after 10 days at 30°.

Fig. 1. Na_2EDTA 10^{-3} M (× 1.)

Fig. 2. Trans-1,2-diaminocyclophexane-*N,N,N',N'*-tetraacetic acid 10^{-3} M. (× 1.)

Fig. 3. Control. (× 1.)

Fig. 4. Phenylthiourea 10^{-3} M. (1.)



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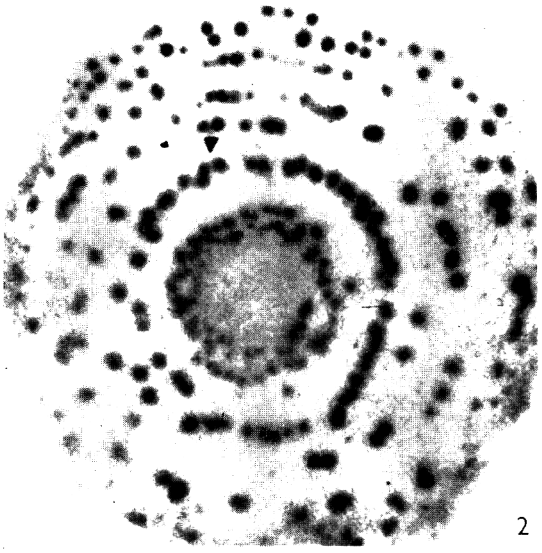

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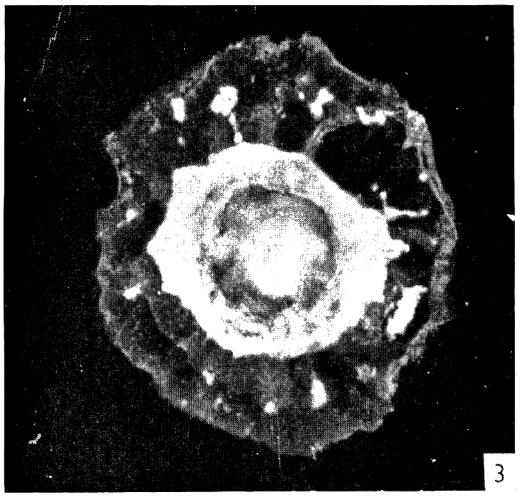

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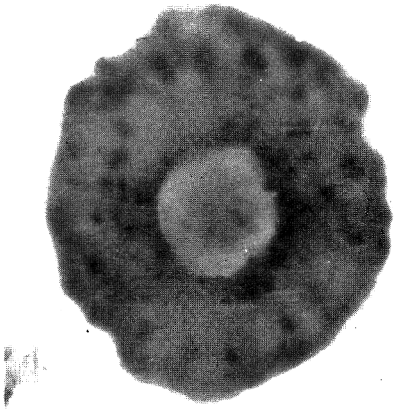

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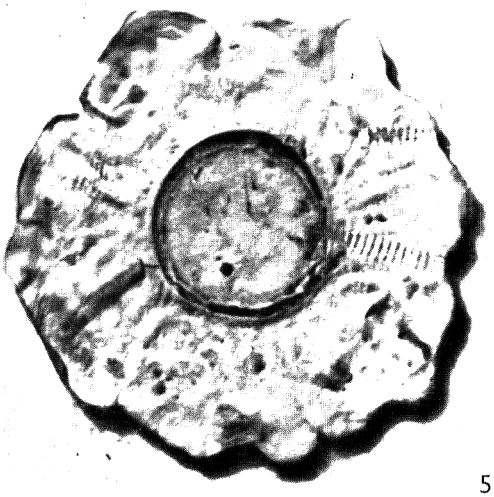

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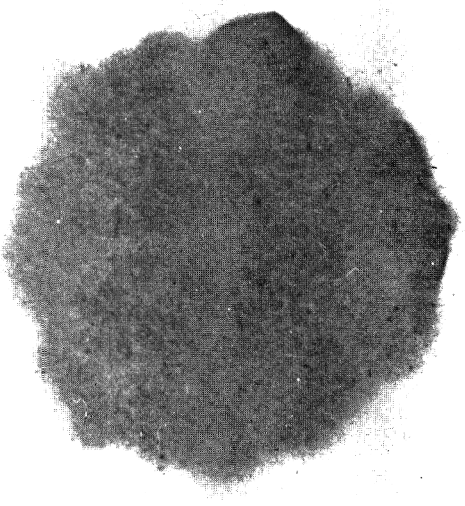

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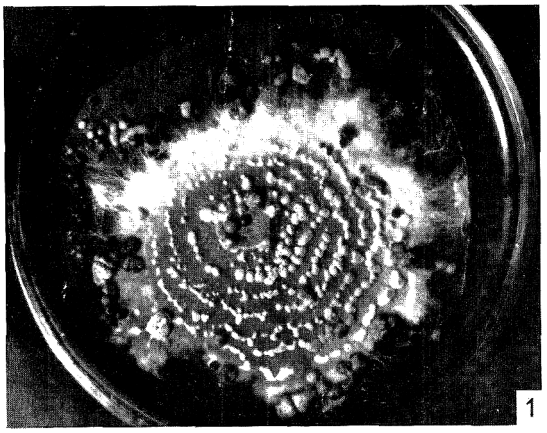
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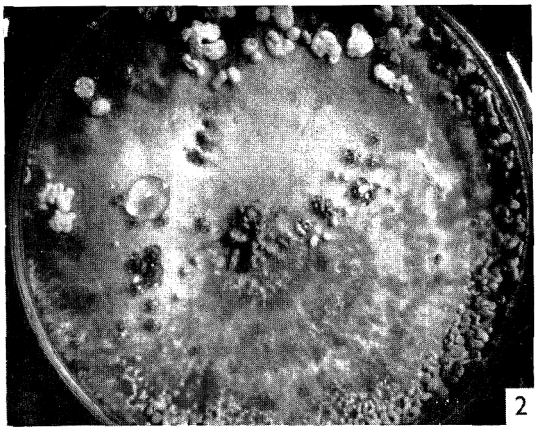
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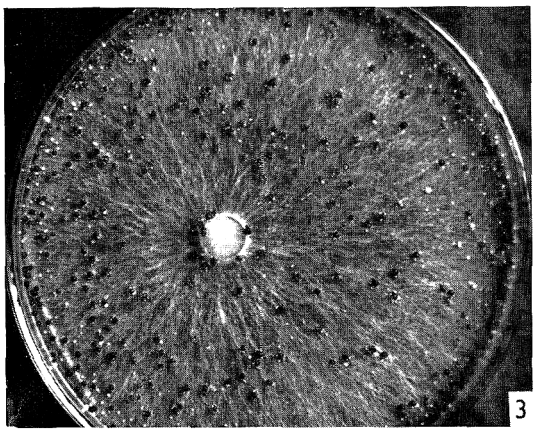
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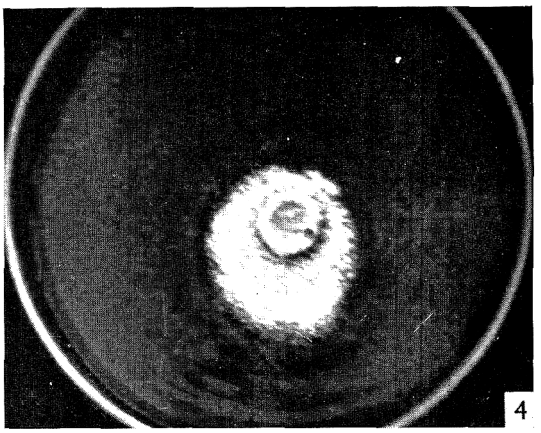
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Antigenic Components of the Cell Wall of *Streptococcus salivarius*

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SUMMARY

A survey of 91 strains of *Streptococcus salivarius* showed that 72 could be classified as type I or II. Only type I strains reacted with group K antiserum. The component responsible for type specificity is a cell-wall polysaccharide composed in each case of galactose, glucose, rhamnose and a trace of glucosamine. However, differences in polysaccharide structure are indicated by differences in the rate of release of soluble carbohydrate by dilute acid. Soluble cell-wall products were obtained by digesting wall with a Streptomyces enzyme preparation and used for serological studies. Galactose was the most effective monosaccharide inhibitor of both type I and type II precipitation. Further investigation suggested that type I specificity depends on the grouping *O*-β-D-galactopyranosyl-(1 $\rightarrow$ 6)-D-galactose.

INTRODUCTION

Most strains of streptococci and lactobacilli can be classified into groups by the Lancefield (1933) technique of extraction of serologically reactive material with hot dilute acid. Strains of *Streptococcus salivarius* do not form a single group by this criterion, but have been grouped together because of their ability to form a levan when grown on sucrose agar (Williams, 1956). Sherman, Niven & Smiley (1943) prepared antisera to a number of strains of *S. salivarius* and found that 40% of strains tested were serologically related. Mirick, Thomas, Curnen & Horsfall (1944), by using aqueous and alkaline extracts of encapsulated organisms, divided the strains into two types, I and II. Horsfall (1951) suggested that specific capsular polysaccharides, as distinct from levans, were responsible for serological reactivity and identified at least six serological types. More recent studies have shown that extracts from certain strains of *S. salivarius* react with antisera to group K streptococci (Williams, 1956; Stewart & McKeever, 1963), and that antiserum to type I *S. salivarius* reacts with a type antigen from some group F streptococci (Willers, Ottens & Michel, 1964). The present report describes the isolation and characterization of serologically reactive cell-wall components from strains of *S. salivarius*.

METHODS

Strains of Streptococcus salivarius. A type I strain, designated *Streptococcus hominis*, NCTC 8606, was obtained from the National Collection of Type Cultures, Colindale, London, and a type II strain, designated ATCC 13419, from the American Type Culture

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Collection. Strain 31 was provided by the School of Microbiology, University of Melbourne. A further 91 strains of *S. salivarius* were isolated from human saliva; one chosen for more detailed investigation was designated HCS. These strains were identified by a mucoid growth on agar containing 5% (w/v) sucrose + 0·00025% (w/v) crystal violet + 0·001% (w/v) potassium tellurite + 5% (v/v) horse serum; identification was confirmed by a series of fermentation reactions kindly performed by Mr H. C. Spies of this Institute.

Culture conditions. Cultures for the preparation of cell walls, for inoculation into rabbits and for acid extraction of serologically reactive material were grown at 37° for 18 hr in Trypticase-Soy broth (Baltimore Biological Laboratories, Baltimore, Md., U.S.A.). For testing capsule production, organisms of strain NCTC 8606 were grown in 1 l. of each of the following media: Trypticase-Soy broth (referred to as M 1), Todd-Hewitt broth (M 2), and the diffusible components of Todd-Hewitt broth (M 3). After 18 hr at 37°, organisms were removed by centrifugation, washed twice with 0·85% (w/v) NaCl and negatively stained with Indian ink to detect capsules. The culture fluid and washings were retained.

Cell-wall preparation. Organisms were harvested by centrifugation, washed, and shaken with Ballotini no. 13 glass beads in the attachment for the International centrifuge (Shockman, Kolb & Toennies, 1957), until the Gram reaction indicated that at least 95% disintegration had been effected. Following differential centrifugation to remove unbroken organisms and soluble cytoplasmic contents, the crude cell-wall preparation was heated for 30 min. at 100°, and incubated overnight at 37° with trypsin + ribonuclease (Cummins & Harris, 1956). The preparation was washed three times with 0·05 M-tris + HCl buffer (pH 7·8), then with water until free from material with absorption maxima at 260 mμ (nucleic acid) and 280 mμ (protein), and dried from the frozen state.

Soluble cell-wall fractions. In preliminary experiments preparations of cell wall from strains NCTC 8606, ATCC 13419, 31 and HCS were incubated with lysozyme and the muralytic enzyme(s) extracted from the growth medium of *Streptomyces albus* (McCarty, 1952). Lysozyme had no detectable action, whereas the Streptomyces enzyme caused a partial dissolution. Consequently cell-wall preparations from each of the above four strains were suspended in 0·05 M-tris + HCl buffer (pH 7·8) to a final concentration of 0·3% (w/v), and 0·05% (v/v) of Streptomyces enzyme preparation added. After 44 hr at 37°, the extinction at 600 mμ had fallen to 10% of its original value. Cell-wall residue was removed by centrifugation and the supernatant solution fractionated with $(NH_4)_2SO_4$ (Knox, 1963); the material precipitated by 70% saturation was designated Fraction A, and that remaining in solution Fraction B.

Serology. Antisera to strains NCTC 8606, ATCC 13419, 31 and HCS were prepared by the procedure used by Knox & Brandsen (1962), with and without the use of adjuvant. Antiserum to group K streptococci was obtained from Burroughs Wellcome, United Kingdom.

Acid extracts of organisms were prepared by the method of Lancefield (1933). Antibody reactions were detected by the precipitin ring test and its micro-modification in capillaries (Swift, Wilson & Lancefield, 1943), by double diffusion in agar (Ouchterlony, 1953), and by quantitative estimation of the precipitate formed (Heidelberger & MacPherson, 1943).

Carbohydrates. *O*-β-D-Glucopyranosyl-(1 → 6)-D-galactose was prepared as

described previously (Knox, 1965). Professor K. Freudenberg (University of Heidelberg, Germany) kindly provided *O*-β-D-galactosyl-(1 → 6)-D-galactose; and *O*-β-D-glucosyl-(1 → 3)-D-galactose (solabiose) was the generous gift of Professor R. Kuhn (Max-Planck Institüt, Heidelberg, Germany). All other carbohydrates were commercial preparations.

Paper chromatography. The following solvents were used: *A*, ethyl-acetate + pyridine + water (10 + 4 + 3, by vol.); *B*, butan-1-ol + pyridine + water (6 + 4 + 3 by vol.); *C*, butan-2-one + acetic acid + water saturated with boric acid (9 + 1 + 1, by vol.; Rees & Reynolds, 1958); *D*, propan-1-ol + ammonia + water (6 + 3 + 1, by vol.); *E*, butan-1-ol + acetic acid + water (6 + 1 + 2, by vol.) followed by *F*, pyridine + water (4 + 1, v/v) along the perpendicular axis for separation of amino acids. Chromatograms were developed in a descending direction on Whatman no. 3 MM or Whatman no. 1 paper. Sugars were detected with alkaline silver nitrate (Trevelyan, Procter & Harrison, 1950), alcohols by a modification of this method (Frahn & Mills, 1959), hexosamines with alkaline acetylacetone and Ehrlich's reagent (Partridge, 1948), and amino acids with ninhydrin (0·2 %) in acetone.

Paper electrophoresis. Paper electrophoresis was done on strips of Whatman no. 3 MM paper, 64 cm. long, between horizontal glass plates, 1·4 cm. thick, in 0·05 M-sodium tetraborate (Frahn & Mills, 1959); a potential difference of 800–900 V was applied for 5 hr; cooling was done by doing the experiments in a refrigerator. Carbohydrates were detected with a modified alkaline silver nitrate reagent (Frahn & Mills, 1959).

Analytical methods. Hexose was estimated by the primary cysteine + H_2SO_4 method (Dische, 1955), glucose with glucose oxidase (Huggett & Nixon, 1957) and galactose with galactose oxidase (Roth, Segal & Bertoli, 1965). Rhamnose was estimated by the method of Dische & Shettles (1948) as modified by Gibbons (1955).

Hydrolysis before the estimation of hexosamines was done with 4 N-HCl at 100° for 1 hr. Muramic acid was separated from glucosamine and galactosamine on charcoal + celite columns by the method of J. T. Park, as described by Perkins & Rogers (1959); the hexosamines were then estimated by the method of Rondle & Morgan (1955). Galactosamine was estimated by the method of Cessi & Serafini-Cessi (1963).

Borohydride reduction. Reduction of oligosaccharides (approximately 3 μmoles in 1·0 ml.) was effected with sodium borohydride (25 mg.). After 4 hr at room temperature, the solution was neutralized, de-ionized with Bio-Deminrolit (carbonate form; The Permutit Co. Ltd.), and repeatedly evaporated to dryness from methanol to remove residual borate. After hydrolysis for 2 hr in 2 N-HCl at 100° the products were identified by chromatography in solvent *C*.

Periodate oxidation. Oxidation of oligosaccharides was done by a modification of the method of Kabat & Mayer (1961). The carbohydrate solution (1·0 ml. containing approximately 0·3 μmole) was mixed with 0·5 ml. of 0·004 M-sodium periodate and allowed to stand in the dark at room temperature (20°). At suitable intervals, 0·1 ml. of solution was diluted to 1·0 ml. and the extinction read at 225 mμ; the end-point occurred after 3–4 hr, when no further uptake of periodate was apparent.

RESULTS

Composition of cell components

Analysis of cell-wall products. Acid hydrolysates of the cell-wall preparations from the four strains of *Streptococcus salivarius* were examined by paper chromatography for component carbohydrates; glucose, galactose, rhamnose, glucosamine and muramic acid were detected. The results of quantitative analysis are given in Table 1. Galactosamine was not detected by paper chromatography, nor by the specific method for its estimation (Cessi & Serafini-Cessi, 1963). Table 1 also contains information on the yields and composition of the preparations designated Fraction B; these preparations were obtained in each case by ammonium sulphate fractionation of the products obtained by the action of the Streptomyces enzyme on cell-wall preparations of the appropriate strain. Glucose, galactose and rhamnose were major components of both the cell wall and Fraction B, presumably being components of a polysaccharide or polysaccharides attached to the mucopeptide. Glucosamine and muramic acid are known components of mucopeptide, but the present results do not exclude the possibility that glucosamine may also be a component of the polysaccharide(s). Qualitative amino acid chromatography indicated that only those amino acids were present which might be expected to be components of the mucopeptide, namely; lysine, glutamic acid, aspartic acid and alanine.

Tests for the possible formation of capsule. Organisms of strain NCTC 8606 grown in media M1, M2 and M3 did not possess a capsule demonstrable with indian ink. However, because of the suggested presence of a type-specific capsule (Mirick *et al.* 1944) other tests were done. Organisms were grown in each of the three media, washed and dried from the frozen state. Each of the culture fluids was dialysed, centrifuged and retained for carbohydrate analysis. The yields of organisms per litre of culture fluid were (dry wt): 200 mg. for M1, 140 mg. for M2, 160 mg. for M3. Batches of organisms from 1 l. culture fluid were then subjected to two extraction procedures: shaking at room temperature in 2·5% (w/v) NaCl for 2 hr (Knox & Hall, 1964), and standing in water at room temperature overnight (Mirick *et al.* 1944). The dialysed culture fluids and the extracts were examined quantitatively for the presence of hexose and rhamnose, which had previously been shown to be components of the cell-wall polysaccharide. In no case did the amount of these components exceed 2% of that present in the corresponding organisms. The culture fluids were also tested for ketose with Seliwanoff's reagent; the results were negative, indicating that levan was not present.

Release of carbohydrate from cell walls by mild acid hydrolysis. During the investigation of the serological properties of cell extracts prepared by the Lancefield (1933) method, it was observed that those from type I stains gave a strong reaction, whereas those from type II strains gave a weak, although positive, reaction. It was shown by Knox & Hall (1965 *b*) that when cell walls of *Lactobacillus casei* were subjected to mild acid hydrolysis, the linkage between the specific polysaccharide and the mucopeptide was hydrolysed. Since many streptococci yield a similar serologically reactive polysaccharide, the rate of release of carbohydrate from *Streptococcus salivarius* cell wall by dilute acid was examined. The conditions used were the same as those previously found to be satisfactory for *L. casei*, namely, heating in 0·1 N-H_2SO_4 at 60°. Suspensions of cell wall (1 mg./ml.) from each of the four strains were heated for appropriate

periods, cooled, centrifuged, and the hexose and rhamnose content of the supernatant fluid determined. The results obtained at three selected intervals are compared in Table 2; they are expressed as % of the total amount of hexose and rhamnose in the appropriate cell-wall preparation. The results showed that although the rate of rhamnose release was similar for each strain, the type I strains (NCTC 8606 and 31) gave a much more rapid release of hexose; in 1 hr this was comparable with the amount released from *Lactobacillus casei* cell wall under the same conditions.

To obtain the polysaccharide component of the cell wall with minimal degradation, preparations of cell walls (500 mg.) from strains NCTC 8606 and ATCC 13419 were heated in 0·1 N-H_2SO_4 at 60° for six 30 min. periods. After each period, the suspension was centrifuged, the supernatant fluid removed and the residue re-heated in the original volume. The pooled supernatant solutions were dialysed, and total carbohydrate determined on the diffusible and indiffusible fractions. The analyses indicated that approximately 94% of the carbohydrate was indiffusible. The indiffusible fractions were dried from the frozen state; the yield from NCTC 8606 was 192 mg. and from ATCC 13419 was 88 mg. The components of each preparation were galactose, glucose, rhamnose and a trace of glucosamine (Table 1).

Table 1. *Analyses of cell-wall components of Streptococcus salivarius*

Procedures for the preparation of the fractions are given in the text. Results are expressed as % dry weight of the fraction; –, analysis not performed.

Strain	Fraction	Yield (% of wall)	Analyses				
			Glucose	Galactose	Rhamnose	Glucos-amine	Muramic acid
NCTC 8606	Wall		14·4	17·0	17·5	7·0	9·2
	B*	30	23·5	10·8	29·0	6·7	6·6
	Polysac.†	38	26·0	37·0	23·5	2·5	–
	Residue†	40	7·1	7·0	12·0	–	–
31	Wall		17·5	9·2	21·0	10·0	9·0
	B*	50	26·5	19·9	32·0	6·0	7·0
ATCC 13419	Wall		13·5	20·0	21·0	8·4	9·0
	B*	20	18·5	6·6	32·0	8·2	7·2
	Polysac.†	18	24·9	35·7	22·5	2·0	–
	Residue†	26	5·1	12·5	10·5	–	–
HCS	Wall		10·0	5·6	17·0	10·0	9·5
	B*	30	19·0	14·8	35·0	14·0	5·0

* Purified soluble products obtained from cell walls treated with Streptomyces enzyme.

† Polysaccharide extracted from cell walls by dilute acid, and residue remaining after prolonged extraction.

Cell walls were heated for a further 16 hr, and the residues then recovered, washed and freeze-dried. The residue from each strain still contained significant amounts of polysaccharide (Table 1).

Serological examination of soluble cell components

A serological survey of 91 strains of *Streptococcus salivarius* isolated from human saliva showed that the majority could be classified as either type I or type II: (*a*) 39 strains reacted with type I antiserum only; (*b*) 33 strains reacted with type II antiserum only;

(*c*) 11 strains reacted with both antisera; (*d*) 8 strains reacted with neither antiserum. A reaction with group K antiserum was given by 38 strains in category (*a*) and by all the strains in category (*c*), i.e. 49 of the 50 strains that reacted with type I antiserum.

Table 2. *Streptococcus salivarius. Amounts of hexose and rhamnose released on heating cell walls in 0·1 N-H_2SO_4 at 60°*

Results are expressed as % total amount present in the wall.

	S. salivarius strain							
	NCTC 8606		31		HCS		ATCC 13419	
Time (hr)	Hexose	Rhamnose	Hexose	Rhamnose	Hexose	Rhamnose	Hexose	Rhamnose
1·0	52	29	37	27	7	24	12	13
4·0	75	37	68	40	19	35	22	29
16·0	87	68	79	60	40	58	33	45

Strain 31 cross-reacted with antiserum to strain NCTC 8606 but not with antiserum to strain ATCC 13419, and was therefore concluded to be a type I strain. Lancefield extracts of whole organisms were shown to contain three serologically reactive components when tested against homologous antiserum, group K antiserum or antiserum to strain NCTC 8606. Lancefield extracts of cell wall and Fraction B from strain 31, as well as Fraction B from strain NCTC 8606, contained a single component which appeared to correspond to the strongest of the three bands obtained with extracts of whole organisms.

Strain HCS cross-reacted with antiserum to ATCC 13419 (type II) but not with antiserum to strain NCTC 8606 (type I) and was therefore concluded to be a type II strain. A comparison of Lancefield extracts of whole organisms, cell walls and Fraction B by the Ouchterlony method indicated the presence of the same three components, one major and two minor, in each preparation; similar results were obtained with homologous antiserum and with antiserum to ATCC 13419.

Inhibition of precipitin reaction by component carbohydrates. The ability of glucose, galactose and rhamnose to inhibit the precipitin reaction between preparations of Fraction B from strains HCS, 31 and NCTC 8606 and homologous antiserum was examined. Antiserum (0·2 ml.) was mixed with 25 μmoles of monosaccharide in a final volume of 0·5 ml. After 1 hr at 37° an appropriate amount of Fraction B was added, and the amount of precipitate formed on subsequent standing determined (Heidelberger & MacPherson, 1943). The results, summarized in Table 3, show that galactose was the most effective inhibitor in each case.

Since the serological reaction with Fraction B from type I strains appeared to involve only one antigenic component, further studies were made of the specificity of the reaction. Cell walls from type I strains were subjected to mild acid hydrolysis and the oligosaccharide components separated (see below). Three disaccharides were obtained, but only one contained a non-reducing galactose residue, viz. *O*-β-D-galactopyranosyl-(1 → 6)-D-galactose; 10 μmoles of this disaccharide inhibited the precipitation of Fraction B from NCTC 8606 by 37%. Another product of hydrolysis was identified as *O*-β-D-glucopyranosyl-(1 → 6)-D-galactose. This disaccharide is responsible for the specificity of the group antigen of *Lactobacillus casei*, Group C (Knox & Hall, 1965 *a*). However, qualitative precipitin tests indicate very little, if any, cross-reaction between *L. casei* and *Streptococcus salivarius*.

Identification of disaccharides obtained on partial hydrolysis of cell wall

Preliminary tests showed that the maximum production of oligosaccharides occurred when samples of cell walls were heated in 0·1 N-H_2SO_4 at 100° for 6 hr. Cell walls from strains 31 and 8606 yielded products which were indistinguishable on chromatography in solvent *A*, not only with regard to mobility, but also apparently with regard to the proportion of each present. Cell wall (275 mg.) from strain 31 was hydrolysed under the above conditions and neutralized with barium hydroxide. Oligosaccharides were separated by elution from charcoal+celite followed by preparative paper chromatography in solvent *A*, as described previously (Knox & Hall, 1965 *a*). Three presumptive disaccharides were obtained, the yields being calculated from the hexose content: D_1, 0·4 mg.; D_2, 1·5 mg.; D_3, 5·9 mg.

Table 3. *Streptococcus salivarius. Inhibition of precipitin reaction between preparations of Fraction B and homologous antiserum by 20 μmoles of monosaccharide*

Serological type	Source of Fraction II	Amount (μg.)	% inhibition by		
			Glucose	Galactose	Rhamnose
I	Strain 31	20	7	40	20
I	Strain NCTC 8606	40	7	23	5
II	Strain HCS	20	9	40	17

Identification of compound D_1 as O-β-D-galactopyranosyl-(1 → 6)-D-galactose. Only one component was detected by chromatography in solvents *A* (R_{Glc}: 0·34) and *B* (R_{Glc}: 0·39), and electrophoresis in sodium tetraborate (M_G: 0·80). Acid hydrolysis followed by chromatography in solvents *A* and *B* showed galactose to be the only monosaccharide component. A 0·02% (w/v) solution of D_1 (0·2 ml.) was incubated with 1% (w/v) almond emulsin (0·1 ml.) in 0·01 M-acetate buffer (pH 5·0) at 37° for 18 hr. After de-ionization, paper chromatography in solvent *A* showed that partial hydrolysis of the compound had occurred (> 50%), to yield galactose as the only product. A sample of D_1 (0·3 μmole) was oxidized with sodium metaperiodate for 4 hr. The amount of periodate consumed was 4·9 moles/mole, which is consistent with a 1 → 6 glycosidic linkage (theoretical consumption = 5 moles/mole). The chromatographic and electrophoretic mobilities are also consistent with a 1 → 6 linkage. Further, compound D_1 was indistinguishable from an authentic sample of *O*-β-D-galactopyranosyl-(1 → 6)-D-galactose on chromatography in solvent *A* and on electrophoresis in borate buffer.

Identification of compound D_2 as O-β-D-glucopyranosyl-(1 → 6)-D-galactose. Only one component was detected on chromatography in solvents *A* (R_{Glc}: 0·39) and *B* (R_{Glc}: 0·45), and on electrophoresis in sodium tetraborate (M_G: 0·70). Acid hydrolysis and almond emulsin both yielded apparently equal amounts of glucose and galactose, as detected by paper chromatography in solvent *A*. The terminal reducing sugar was determined by reduction with sodium borohydride followed by acid hydrolysis and chromatography in solvent *C*; the products were glucose and galactitol. Compound D_2 was indistinguishable from *O*-β-D-glucopyranosyl-(1 → 6)-D-galactose (Knox, 1965) on chromatography in solvents *A* and *B*, and on electrophoresis in sodium tetraborate.

Identification of compound D_3 as O-β-D-glucopyranosyl-(1 → 3)-D-galactose. This was the major product of acid hydrolysis; only one compound was detected on electrophoresis in sodium tetraborate (M_G: 0·48) and chromatography in solvents *A* and *B* (R_{Glc}: 0·56, 0·59, respectively). Acid hydrolysis and almond emulsin (100% hydrolysis) both yielded apparently equal amounts of glucose and galactose, as detected by chromatography in solvent *A*. When acid hydrolysis was preceded by reduction with sodium borohydride, the products detected were glucose and galactitol. Disaccharide D_3 consumed 2·8 moles/mole of sodium metaperiodate, consistent with a 1 → 2 or a 1 → 3 glycosidic linkage (theoretical consumption = 3 moles/mole). The possibility of a 1 → 2 linkage was excluded since compound D_3 formed a red formazan with triphenyl tetrazolium chloride (Wallenfels, 1950). The $[\alpha]_D^{27.5}$ of a solution of D_3 (*c*: 0·17 %) was $+20° \pm 5°$. Because of the low concentration, this value was not regarded as sufficiently accurate for identification, but is consistent with a β-linkage; the reported value for this disaccharide is $+35°$ to $+40°$ (Bailey, 1965).

DISCUSSION

In confirmation of the results of previous workers (Mirick *et al.* 1944; Horsfall, 1951; Williamson, 1964), it has been found that most strains of *Streptococcus salivarius* can be classified as either type I or type II. The known reaction of certain strains of *S. salivarius* with group K antiserum (Williams, 1956; Stewart & McKeever, 1963) resides exclusively with the type I strains, 98% of which reacted with group K antiserum. Williamson (1964), who obtained cultures from throat swabs, classified 78% of the strains as type I and 6% as type II. In the present investigation, where organisms were isolated from saliva, the corresponding values were 43 and 36%. It has been shown that the tongue is the source of oral strains of *S. salivarius* (Gibbons, Kapsimalis & Socransky, 1964), so that these differences in relative proportions may reflect the different sources of the strains.

It is apparent that cell-wall polysaccharides account for types I and II specificity. In each case the major components are galactose, glucose and rhamnose, with glucosamine (a major component of the mucopeptide) being a minor component of the polysaccharide. The finding of the three sugars in the wall of strain NCTC 8606 confirms the earlier observation by Colman & Williams (1965). Carbohydrate analyses of cell-wall preparations do not indicate differences which could account for the differences in serological specificity. However, as shown in Table 2, there are differences in the rates of release of rhamnose and hexose from cell wall preparations from types I and II organisms, and this probably reflects differences in structure.

The partial release of polysaccharide by dilute acid enabled the Lancefield procedure to be used for differentiating the two types. However, the observation that release of polysaccharide was incomplete even after 16 hr, contrasts with the results obtained with *Lactobacillus casei* (Knox & Hall, 1965 *b*) and *L. fermenti* (Knox & Holmwood, 1968). With the lactobacilli, rapid release was shown to depend on the hydrolysis of a phosphodiester linkage between the polysaccharide and mucopeptide components. The results with *Streptococcus salivarius* suggest, therefore, that part at least of the polysaccharide component may be joined to the mucopeptide by a linkage other than a phosphodiester.

A comparison of the carbohydrate analyses of cell walls, the polysaccharide com-

ponents and the products obtained by Streptomyces action would also suggest that for both types I and II, the cell walls contain polysaccharide chains of varying composition. An indication that the polysaccharide fraction from type II cell wall does contain different components was provided by the results of the Ouchterlony procedure, where one major and two minor components were detected. However, with type I cell wall, the product of Streptomyces action contained only one serologically reactive component.

Owing to the impurity of the preparation, the examination of type II specificity was limited to the inhibition of the precipitin reaction by component sugars; these studies indicated that galactose was the immuno-dominant sugar. Studies on the type I specificity indicated that, in this case also, galactose was the immuno-dominant sugar, and this supported the results of Willers *et al.* (1964). From further studies on the inhibition by glucosides, Willers *et al.* (1964) concluded that the antigenic determinant was probably a β-1 → 6-glucoside, though the ability of galactosides to inhibit the precipitin reaction was not tested. The present study enabled the inhibition by a galactoside to be examined; from the products of hydrolysis of cell wall *O*-β-D-galactopyranosyl-(1 → 6)-D-galactose was isolated and shown to be a very effective inhibitor of the precipitin reaction. It is therefore concluded that this disaccharide constitutes part of the antigenic determinant for type I specificity.

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Factors Influencing the Extent of Germination of *Bacillus coagulans* Spore Populations

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SUMMARY

The rate of germination of populations of spores of *Bacillus coagulans* decreased as germination progressed, late-germinating spores exhibiting a delay or decreased probability of germination. Delayed germination was not due to changes occurring in the environment during germination. Colonies formed by delayed individuals had a similar morphology to those produced by the rest of the population. Delayed individuals constituted not more than 5% of the total spore population. In a sample containing 17 delayed individuals, 14 gave spore populations with initial germination rates like that of the parent population, while three yielded populations which germinated at much slower rates. Delayed germination still occurred in spore populations grown from single colonies of the parent population. It was concluded that the spore populations were heterogeneous with regard to germination; reasons for the heterogeneity are discussed.

INTRODUCTION

Heterogeneity of spore populations has been noted or suspected in the phenomena of germination (Schmidt, 1955; Hyatt & Levinson, 1961; Foerster & Foster, 1966; Wax, Freese & Cashel, 1967), heat activation (Busta & Ordal, 1964) and ethylene oxide resistance (Church, Halvorson, Ramsey & Hartman, 1956). The non-logarithmic curve of extinction-decrease of spore suspensions during germination (McCormick, 1964, 1965) was interpreted as being due to the heterogeneity of the spores (Vary & McCormick, 1965; Vary & Halvorson, 1965). Further evidence for germination heterogeneity is presented here.

When a bacterial spore population in a constant germination environment exhibits a logarithmic decline in spore numbers due to germination it can be concluded that each spore has an equal chance of germinating, regardless of length of exposure to the germinant. However, when spore populations germinate, even under approaching constant conditions, some spores may still be detected long after most of the spores have germinated. When such ungerminated spores appear with greater frequency than expected from a logarithmic decline in spore numbers, they may justifiably be called delayed germinators. This paper reports experiments confirming the existence of delayed germinators and assesses the relative importance of environment and intrinsic factors.

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METHODS

Organisms and spore production. The organism used was *Bacillus coagulans* NCTC 3991, *B. cereus* T was also briefly examined. The parent population of *B. coagulans* spores had been stored at 4° for 2 years in distilled water. It was produced as described previously (Hitchins, Gould & Hurst, 1963).

Spore populations were derived from delayed germinators as follows. Spores of the parent population were incubated for 10 days in 10 mM-L-alanine + sodium metabisulphite ($Na_2S_2O_5$) in sodium phosphate buffer (pH 7·0, 100 mM). Solutions of $Na_2S_2O_5$, sterilized by filtration, were added to germination media (0·16% final concentration) to prevent growth of germinated spores; this did not prevent initiation of germination (Gould, 1964). Use of $Na_2S_2O_5$ enabled the course of germination to be monitored for long periods of time. A sample was heated at 70° for 30 min. to kill germinated spores and was found to contain about 0·01% of the original spore concentration. Single colonies derived from these residual spores were obtained by growth on yeast glucose agar. The colonies were subcultivated in nutrient broth aerated by agitation until free spores were again formed. Control spore populations were derived in the same way, but the germination and heating steps were omitted. *Bacillus cereus* T spores were produced in nutrient broth. All spores were grown at 37°. Viability of spores was determined by microscopic observation of a yeast glucose agar slide culture after incubation for 6 hr at 37°.

Media and chemicals. Germination was done in yeast glucose broth (YGB) and solutions of L-alanine (10 mM or as indicated) in phosphate buffer (pH 7·0, 100 mM). *Bacillus cereus* spores were germinated in solutions of L-alanine (10 mM) or inosine (10 mM) in buffer. *O*-carbamyl-D-serine (OCDS) was the gift of P. H. Hidy (Commercial Solvents Corp., Terre Haute, Ind., U.S.A.). It was used at 2·4 mM in germination media. Nutrient agar and sometimes yeast glucose agar were used for colony counts.

Germination. Spores were activated by heating to 70° for 30 min., unless otherwise stated. Throughout this paper the term germination refers solely to the initiation stage of germination during which heat resistance is lost. The germination temperature was always 37° and the initial spore concentration was about 10^6 spores/ml., except as indicated. Germination kinetics were studied by counting the number of colony-forming units which survived heating at 70° for 15 min. or sometimes 10 min. Samples were decimally diluted in sterile distilled water and pour-plates of appropriate dilutions prepared in duplicate. The plates were incubated at 37° for 2 days. Longer incubations did not give significantly increased counts. The extent of germination was measured by counting the number of heat-resistant colony-forming units remaining in 10 mM-L-alanine + $Na_2S_2O_5$ in sodium phosphate buffer (pH 7·0, 100 mM) after 17 hr.

Preparation of delayed germinators. Spores were added to warmed YGB containing $Na_2S_2O_5$ to a final concentration of about 3×10^6 spores/ml. The course of germination was followed by viable-spore colony counts. After 3 hr the spore suspension was washed twice with cold sterile distilled water and heated at 70° for 15 min. to kill germinated spores. The resulting suspension was tested for rate of germination in fresh YGB without further heating.

Time course for appearance of delayed germinators. The course of sporulation in shake flasks of nutrient broth was followed by viable-spore colony counts. Samples (10 ml.) were heated (70° for 30 min.) to kill vegetative forms and the suspensions

washed four times by centrifugation in cold sterile distilled water. The samples, containing spores still in sporangia, were tested for extent of germination. The inoculum concentrations were not more than 5×10^6 spores/ml. and were generally about 10^6/ml.

RESULTS

Viability

Spores (350) from the parent *Bacillus coagulans* population were examined by slide culture; all were found to be viable. Viable counts of the parent population were unaffected by heating at 70° up to 2 hr or by the nature of the plating medium.

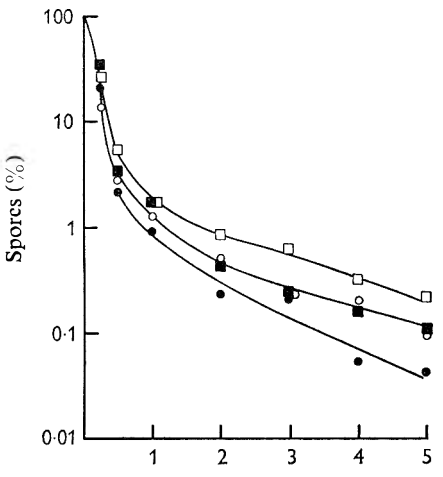

Fig. 1

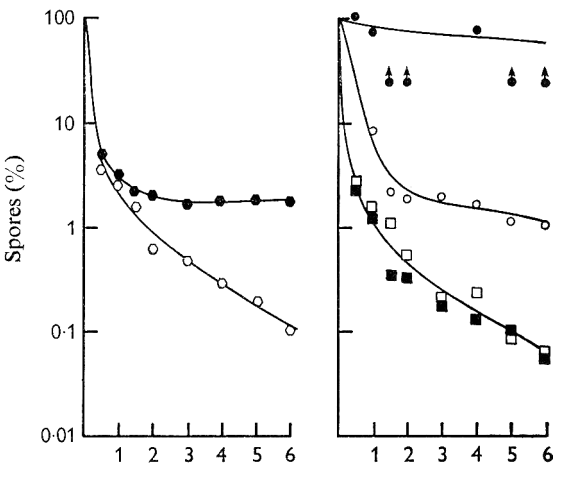

Fig. 2

Fig. 1. Germination of *Bacillus coagulans* spores in YGB as affected by preheating and $Na_2S_2O_5$. ○, preheat only; ■, $Na_2S_2O_5$ only; ●, control: no preheat, no $Na_2S_2O_5$; □, preheat + $Na_2S_2O_5$.

Fig. 2. Effect of *O*-carbamyl-D-serine (OCDS) and spore concentration on *Bacillus coagulans* spore germination in YGB + $Na_2S_2O_5$. Closed symbols, no addition; open symbols, with OCDS. Initial spore concentrations: ●, ○, 3×10^8/ml.; ⬢, ⬡, 3×10^7/ml.; ■, □, 3×10^6/ml. Samples held at 70° for 10 min. to kill germinated spores. Spores were not preheated. Symbols with arrows represent minimum estimates.

Germination kinetics

Figure 1 shows that the germination kinetics of *Bacillus coagulans* spores were non-logarithmic and that there was an initial phase during which the majority of the population germinated rapidly, followed by a later phase of slow germination involving less than 5% of the initial population. The spores that germinated during the later phase thus showed a delay of germination as compared with the majority of spores in the population. Preheating and $Na_2S_2O_5$, although slightly inhibitory to germination, did not alter the typical non-linear shape of the germination curve. Since preheating generally stimulates the rate of spore germination the inhibitory effect was unusual, but was not investigated. Colonial morphology did not vary with sampling time during germination. Heat-shocked *B. cereus* T spores also germinated in a non-logarithmic fashion in L-alanine, inosine or YGB.

Auto-inhibition of germination

Figure 2 shows that germination was slower and less complete at high initial spore concentrations than at low ones. Also Fig. 2 shows that this auto-inhibitory effect was counteracted by the alanine racemase inhibitor OCDS (Lynch & Neuhaus, 1966). OCDS stimulates germination by inhibiting spore alanine racemase (Gould, 1966), which catalyses production of the germination inhibitor D-alanine from L-alanine. The lack of effect of OCDS at the lowest spore concentration indicated that there was no auto-inhibitory effect due to D-alanine; however, delayed germination still occurred. The auto-inhibitory effect is shown further in Fig. 3, in which the % delayed germinators is plotted against the total spore population. Above 10^6 spores/ml. the extent of germination decreased with increasing concentration, whilst below 10^6/ml. the extent of germination was independent of inoculum concentration.

Effect of replacing the medium on the germination kinetics

Two decimal dilutions of a germinating suspension at 10 and 20 min. with fresh warmed medium did not alter the typical non-logarithmic shape of the curve. Initial spore concentration was only $2{\cdot}5 \times 10^6$/ml. and the final concentration of spores + germinated spores after dilutions was only $2{\cdot}5 \times 10^4$/ml. Environmental changes induced by the germinating spores such as those causing auto-inhibition should have been negligible. Furthermore, any inhibitors of germination produced should have been diluted by the fresh medium and counteracted by the high L-alanine concentration (23·2 mM). Nevertheless, delayed germination was still apparent.

Delayed germinators obtained from a germinated spore suspension and representing about 0·2% of the initial population exhibited a logarithmic rate of germination that resulted in about 70% germination in 6 hr. Since the delayed germinators germinated slowly in fresh medium, the delay did not seem to be due to environmental changes occurring during germination.

Effect of concentration of medium constituents and preheating on the extent of germination

When the L-alanine concentration was kept constant (10 mM) the size of the ungerminated fraction increased from 0·02 to 0·30% with increase of the total molarity of the defined medium constituents from 0·07 to 0·50 M. At a buffer concentration of 100 mM, increasing the L-alanine concentration in the range 0·1 to 10 mM decreased the ungerminated fraction from 1·0 to 0·02%. The ungerminated fraction increased to 1·0% when the L-alanine concentration was further increased to 200 mM, presumably because of the high molarity of the medium.

Preheating of spores did not alter the general shape of the germination curve (Fig. 1). The extent of germination was not affected by preheating at temperatures below 80° but at about 88° preheating decreased the extent of germination (Table 1). The apparent killing of spores at the highest temperature was confirmed in another experiment. During preheating at 83° for 80 min. there was a slight decrease in the extent of germination during the first 15 min. similar in size to that which occurred at 81° for 30 min. (Table 1), but after that there was no further change. The increased fraction of ungerminated spores observed after extreme preheating was probably due to a

slower rate of germination caused by heat injury (Busta, 1967). It was concluded that preheating below 80° does not cause delayed germination.

Time of appearance of delayed germinators during sporulation

Experiment 1 in Fig. 4 shows that the extent of germination was always incomplete and that delayed germinators formed a constant proportion no matter at what time the samples were taken during sporulation. Experiment 2 confirmed this result but differed in that the extent of germination was markedly less than in Expt. 1. This was possibly related to a lower rate of aeration, since the rate of appearance of heat-resistant spores was slower and the spore yield was lower than in Expt. 1. Furthermore, considerable lysis of vegetative forms occurred during sporulation in Expt. 2. The results suggested that appearance of delayed germinators was not a function of the stage of sporulation.

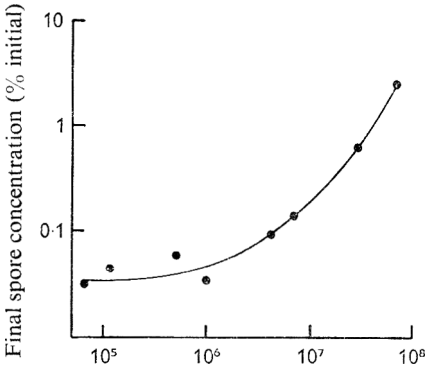

Fig. 3

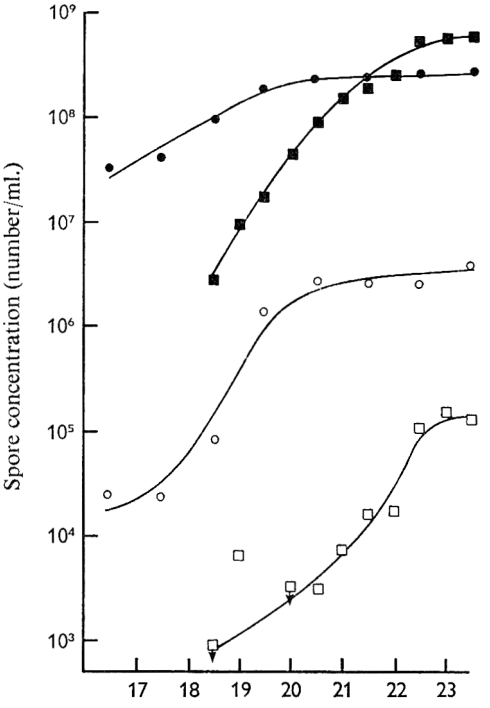

Fig. 4

Fig. 3. *Bacillus coagulans.* Relationship between the initial spore concentration and the size of the ungerminated spore fraction.

Fig. 4. *Bacillus coagulans.* Appearance of heat-resistant spores during sporulation. Closed symbols: total spore population. Open symbols: delayed germinators. Experiments: 1, ■, □; 2, ●, ○. Symbols with arrows represent maximum estimates since the plates had less than 10 colonies.

Effect of subculture on the presence of delayed germinators

Table 2 shows that delayed germinators yielded spore populations which germinated in most cases as quickly as unselected populations. Only three out of 17 delayed

germinators yielded populations with very slow rates of germination. An unselected spore population derived from a colony of the parent stock and a fast-germinating population derived from a delayed germinator were found to have germination curves similar in shape to that of the parent population.

Table 1. *Effect of preheating on the extent of germination of spores of Bacillus coagulans* NCTC *3991*

Preheating		Viability after preheating (%)†	Ungerminated spores (%)‡
Temp. (°)*	Time (min.)		
4	—	100	0·047
70	30	100	0·033
75	30	100	0·040
81	30	100	0·074
88	15	100	1·41
88	30	30	7·97

* Temperatures above 80° were nominal values due to fluctuations.
† Based on an initial viable spore concentration of $1{\cdot}23 \times 10^6$/ml.
‡ Based on the concentration of viable preheated spores.

Table 2. *Bacillus coagulans* NCTC *3991. Germination times of spore strains derived from the parent suspension and its 0·01 % ungerminated spore fraction*

Germination time (min.)*	% of strains	
	From parent population	From 0·01% fraction
Less than 3	0	0
3 to 5	8	12
6 to 10	69	53
11 to 20	23	12
21 to 30	0	6
31 to 60	0	0
61 to 76	0	17
Greater than 76	0	0
Number of strains tested	13	17

* Time taken for the extinction to decrease to 75% of its initial value, that for the parent suspension being 7 min.

DISCUSSION

Since delayed germination was not eliminated by attempts to maintain a constant germination environment it can be concluded that it was due to the nature of the delayed germinators. If germination rate can be regarded as a measure of the degree of dormancy, then, although all the spores in the population were dormant, some were more dormant than others. The remarks in this paper only apply to spores that germinated and formed colonies within 48 hr. Spores exhibiting even greater degrees of dormancy would have remained undetected. However, the high viability of the stock spore suspension suggests that such spores, if present, were rare. Since germination heterogeneity was apparent in both simple germinant solutions and in yeast glucose broth, it seems that it was not due to a qualitative difference of germination requirements between fast and slow germinators.

The only detectable factor that altered the constancy of the germination environment in this study was the auto-inhibitory effect of high spore concentrations (Fig. 2) which has been described previously (Stedman, Kravitz, Anmuth & Harding, 1956; Stedman, Kravitz, Harding & King, 1957). Auto-inhibition of germination is due to D-alanine being produced from the germinant L-alanine (Fey, Gould & Hitchins, 1964) by alanine racemase. D-Alanine is a well-known inhibitor of spore germination and it acts by inhibiting L-alanine-induced germination (O'Connor & Halvorson, 1961). *O*-carbamyl-D-serine (OCDS) inhibits alanine racemase (Lynch & Neuhaus, 1966) and its stimulating effect on germination is due to its inhibition of spore alanine racemase (Gould, 1966). Since the auto-inhibitory effect with *B. coagulans* was counteracted by OCDS it was probably due to the action of spore alanine racemase.

Woese, Vary & Halvorson (1968) suggested that the non-logarithmic germination curve might be due to a Poisson distribution of a germination enzyme, which might be L-alanine dehydrogenase (Halvorson, Vary & Steinberg, 1966) or perhaps the protein initiator of germination isolated by Vary & Halvorson (1968). This would mean that the distribution of germination heterogeneity in the population is continuous. However, the results presented here could be interpreted as meaning that the germination heterogeneity is discontinuous, the population consisting of a mixture of two variants, the minority variant forming the delayed germination spore fraction. Unfortunately it is difficult to decide between the possibilities of a dichotomously or continuously variable population (Gilbert *et al.* 1964).

With *Bacillus cereus* spores delayed germination was apparent in the presence of the two germinants L-alanine and inosine. L-Alanine dehydrogenase is almost certainly not involved in inosine germination. This suggests that the variation of some spore component other than L-alanine dehydrogenase, but having a common relationship to the mechanisms of germinative action of both germinants, was responsible for the germination heterogeneity. Whatever the nature of the variable spore component, it was not affected by environmental conditions during spore formation by *B. coagulans*. The fast- and slow-germinating spores both developed heat resistance at about the same time during sporulation, the slow ones not appearing markedly earlier or later than the fast ones.

Some of the delayed germinators may have been slow-germination mutants since they formed slow-germinating spore populations, but the majority formed fast-germinating spore populations. This may mean that if delayed germination was due to mutation, there might have been a high rate of reversion. At least some of the fast-germinating populations obtained from either fast or slow germinators were heterogeneous, since they also contained some delayed germinators, suggesting that the ability to produced heterogeneous populations is inheritable. Germination heterogeneity resembles in this respect the dissociation phenomena characteristic of the genus *Bacillus* (Braun, 1947; Smith, Gordon & Clark, 1952; Moore, 1965) or the quantitative mode of inheritance of oligosporogeny (Northrop & Slepecky, 1967). Alternatively, the apparent inheritability of heterogeneity is not inconsistent with a non-genetic explanation such as that of Woese *et al.* 1968. The relative importance of the various possibilities remains to be decided.

I am grateful to Dr G. W. Gould and Dr W. A. Hamilton for helpful criticism during the preparation of this paper.

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Viable Counts of Bacteria—a New Method for Facultative Anaerobes

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SUMMARY

Large dilutions of bacterial suspensions were made at 45° in a buffered complex medium containing agar. The tubes of medium with the serial dilutions of bacteria were solidified by cooling; subsequent incubation at 37° permitted bacterial growth in discrete colonies. The density of colonial growth was enumerated by comparison with a set of prepared standards calibrated by the pour-plate technique. A large range ($\log_{10}$ 2·5 to 10·5) of colony counts was covered in only four test tubes without any prediction of the approximate count. The method has advantages of speed, economy of reagents and apparatus, as well as range and precision.

INTRODUCTION

There are many methods for the assessment of the number of viable bacteria in a population (Meynell & Meynell, 1965). In direct methods, a suspension containing a suitable number of bacteria is transferred to a solid nutrient medium which, under suitable conditions, permits each viable bacterium to grow to a colony. Direct count of the colonies and reference to the dilution of the original suspension yield the colony count. This type of method is generally satisfactory, but effort wasted in plating unsuitable dilutions can only be avoided to the extent that the order of magnitude of the result is predictable. The present paper describes a radically new approach which has advantages over other methods. Without loss of accuracy, it is more economical in the use of media and apparatus (especially graduated glassware), does not require a prediction of the colony count of the unknown, and does not demand the error-prone and tedious counting of thousands of colonies or mathematical processing of results. The method has only been applied to facultative anaerobes, cannot be used for aerobes, and would probably require modification for use with obligate anaerobes.

METHODS

Bacterial strain and media. Escherichia coli ATCC 11229 was grown on an orbital shaker (L.H. Engineering Co., Bells Hill, Stoke Poges, Bucks.) at 37° in a defined medium containing (g./l.): glucose, 12; $MgSO_4.7H_2O$, 0·4; KH_2PO_4, 5·4; $(NH_4)_2SO_4$, 1·2; NaOH to adjust to pH 7·2. Pour-plates for colony counts were made in nutrient agar (Oxoid Ltd., London). Colony counts by the method described in this paper were done in a medium containing (g./l.): tris (2-amino-2-hydroxymethyl-propane-1,3-diol; Sigma, London, Ltd.), 2·017; maleic acid (British Drug Houses lab. reagent), 1·93; peptone (Difco, Baird and Tatlock (London) Ltd.), 30; yeast extract (Difco), 50; agar (Oxoid ionagar no. 2), 6; adjusted to pH 7·2. This medium was sterilized at

108° (5 lb./sq.in. pressure of steam). This buffered complex medium (BCM) was normally stored at 4°, melted in boiling water and 5·9 ml. portions transferred into sterile 150 × 16 mm. rimless tubes which were then stored in water baths at 48° up to 18 hr. It was convenient to cap the test tubes with anodized aluminium caps (Oxoid) and to dispense the molten medium with an automatic syringe pipette (Froud and Sons Ltd., Dalston, London). Where undesignated, chemicals were British Drug Houses Ltd. Analar grade.

Apparatus. Transfers of 0·1 ml. volumes were done with special pipettes (H. J. Elliot Ltd., Pontypridd, Glamorgan) which had been made water-repellent by a silicone treatment. This consisted of immersion in a 1 % (v/v) solution of $SiCl_4$ (M 441, Imperial Chemical Industries Ltd.) in carbon tetrachloride, followed by baking at 110° for 1 hr. Each pipette was fitted with a plastic teat which was controlled by the stainless steel lever carried in the brass pipette holder. The whole arrangement and the manner of operation are shown in Fig. 1. For assembly, the pipette is fitted with a teat and placed in the bottom part of the holder, the lever is located in its slot and the top part pushed home until the whole is firmly held together by friction. The dimensions and arrangements of the holder are not critical, but it should be reasonably light and fit conveniently to the hand. Above all, the lever should permit a high degree of sensitivity with an absolutely positive control.

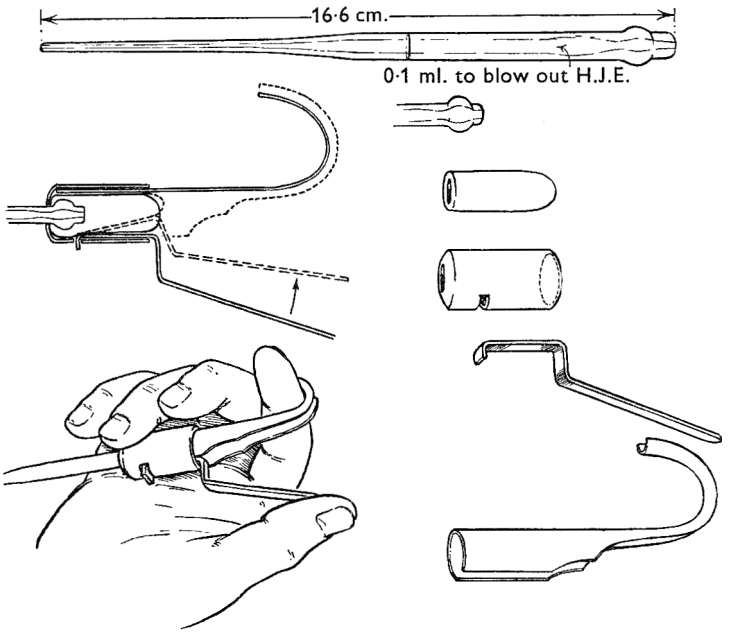

Fig. 1. Arrangement and operation of pipette assembly.

To heat pipettes in boiling water it was found convenient to use a small instrument sterilizer (McCarthys Hospital Equipment, Romford). After transfer to diluent, bacterial suspensions were mixed on a Vortex mixer (Scientific Industries International (U.K.), Ltd.). The growth in the tubes was compared visually under indirect illumination, and it was found convenient to house the set of standards in a viewing-box. The dimensions are not critical and depend on the number of standards

employed. A box which carried 24 standard tubes was 42 in. × 8 in. × 14 in. over-all and was illuminated from below by a 3 ft. 30 W fluorescent tube.

Colony count by pour plates. Serial dilutions for pour plates were made by transfer of 1 ml. portions in serological pipettes to 9 ml. sterile water in capped 150 × 16 mm. rimless tubes. A fresh pipette was used for each dilution and was rinsed three times in both suspension and diluent. Triplicate 1 ml. portions of appropriate dilutions were transferred to sterile, disposable, plastic Petri dishes (Oxoid), thoroughly mixed with about 10 ml. nutrient agar at 48°, allowed to set, and incubated at 37° for 24 hr. The plates were then set against a dark background and the discrete colonies counted. The count was obtained from this value by reference to the serial dilution used.

Colony count by tube dilution. Normally, four 0·1 ml. pipettes were used. Of these, one was in use, two were suspended so that the glass parts were in the sterilizer of boiling water while one was cooling after boiling. A single pipette was used to select the 0·1 ml. sample, transfer it to 5·9 ml. BCM and, after mixing, for successive transfers. The pipette was rinsed in the boiling water between transfers and four tubes of BCM were normally employed although, with *Escherichia coli*, a culture not showing visible turbidity could be assessed in three tubes. When the dilutions were completed the pipette was suspended in the boiling water and the tubes of BCM were placed in cold running water to solidify. With *E. coli* incubation at 37° overnight was usually sufficient to permit maximal colony development, but 40 hr was required for some strains of *Staphylococcus aureus* (unpublished results). Each set of tubes was then compared with the standards. The $\log_{10}$ count was computed by reference to the dilution (1/60, $1/60^2$, $1/60^3$ or $1/60^4$) of the sample and the colony count of the standard with which it was matched.

The standard tubes were prepared so that each tube differed from its immediate neighbours by $\log_{10}$ of 0·1. Since the standards were used over a range much greater than can be directly counted, a method for their preparation was devised. An overnight culture of *Escherichia coli* was diluted 10^{-5} in distilled water and two further dilutions of $10^{-0.1}$ and $10^{-0.2}$ made from this by dilutions of 200/252 and 200/317 respectively. These primary standards were then further diluted by successive factors of $10^{-0.3}$ by adding 100 ml. to 100 ml. sterile distilled water. Each bacterial suspension was assessed for colony count by the pour-plate technique applied in triplicate and was also used to inoculate 24 tubes of BCM. The 'true' count of each member of the dilution series was calculated from *all* of the pour plates and, as this depended on 166,428 colonies selected by 66 independent processes, presumably the sampling error was minimal. There were 24 tubes available for the calibration series at each bacterial concentration. To select the 'best' tube at the most concentrated end of the series, each set of 24 tubes was first graded visually according to density. The middle three tubes (two as spares) from each set were selected and preserved by adding 1 ml. 40% (w/v) formaldehyde (May and Baker Ltd., Dagenham). After standing for 1 week the formaldehyde was changed and the tubes sealed by drawing out in a gas flame. At the lower end of the scale, tubes were selected which were shown by direct count to contain the 'correct' number of colonies 'randomly' distributed in the medium.

Some practice is required in matching an unknown tube to the set of standards on the basis of an over-all visual impression of colony size as well as density. It is the diminution in size of individual colonies, as their number increases, which allows the use of a set of standards covering more than a 100-fold range. Once an unknown tube

has been matched to a standard, the colony count of the culture from which the unknown has been derived is easily calculated from a knowledge of the number of 1/60 dilutions made and the known colony content of the standard. In practice it has proved convenient to construct a table from which the colony count of the unknown is read off directly by reference to the position of the standard in the calibration series and the number of dilutions made.

RESULTS

The validity of the calibration was tested by determination of colony counts with both the pour-plate and tube-dilution methods. Several suspensions of *Escherichia coli* 11229 were prepared by dilution of an overnight culture in water and were deliberately chosen to cover a very wide range of concentrations. The results are shown in Fig. 2. The reproducibility of pour-plate and tube-dilution methods was determined over a large range of bacterial concentrations. Both methods were used to make 10 replicate counts on each of several suspensions; a statistical analysis of the results is given in Table 1.

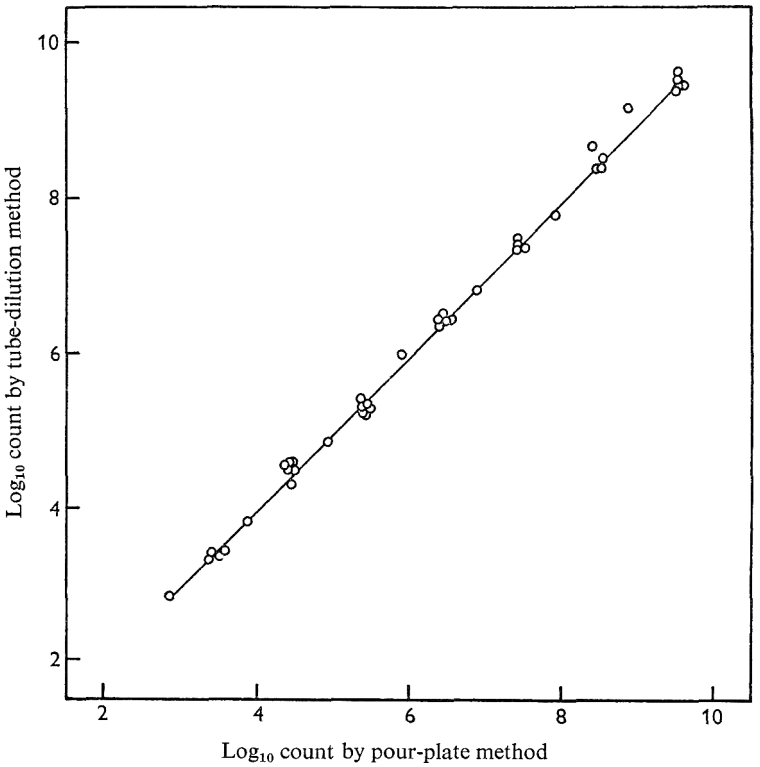

Fig. 2. Comparison of viable count of suspension of *Escherichia coli* by pour-plate and tube-dilution methods.

DISCUSSION

All colony-counting procedures are subject to the errors inherent in sampling a population. This inescapable inaccuracy in sampling is frequently expressed as standard error which is the square root of the number sampled. Clearly, sampling

Table 1. *Colony counts of Escherichia coli by pour-plate and tube methods*

Overnight cultures were diluted in sterile water and ten replicate counts made of each by both counting methods.

Plate count		Tube count	
Mean ($\log_{10}$)	Standard deviation	Mean ($\log_{10}$)	Standard deviation
8·480	±0·157	8·402	±0·059
8·371	±0·056	8·392	±0·053
8·023	±0·107	8·035	±0·154
7·845	±0·077	7·817	±0·022
7·747	±0·098	7·755	±0·041
7·715	±0·162	7·662	±0·056
4·487	±0·071	4·455	±0·069
3·685	±0·034	3·632	±0·089
3·444	±0·045	3·510	±0·032
2·926	±0·087	2·983	±0·022

error, as a percentage of the number sampled, decreases as the number sampled increases. Thus a sample of 50 must have a standard error (S.E.) of about 14% while 100 counts have 10% S.E., 200 counts about 7% S.E., 400 counts about 5% S.E. and 800 counts about 3% S.E. In addition, the actual errors are increased by imperfections in technique so that the

$$\text{total error} = \sqrt{[(\text{technical error})^2 + (\text{sampling error})^2]}$$

(Jennison & Wadsworth, 1940). The data presented here show that the tube-dilution method was somewhat more reliable than the pour-plate technique. However, the count of the 'unknown' suspensions was known sufficiently well to enable the pour plates to be prepared with a final colony count of between 100 and 400. In laboratory practice it is not always possible to do this and the precision of pour plates decreases considerably when fewer colonies are counted. In the tube-dilution method, however, the lowest count which is assessed is usually 600, being 1/60 of the maximum concentration included in the calibration series. Of course the tubes at the higher end of the calibration series do not contain the stipulated density of colonies because of coincidence. The method of calibration allows for this, as is shown by the excellent correlation between tube-dilution and pour-plate methods in Results. In addition, the number of dilutions used is less in tube counts and consequently the technical error should also be less.

As compared with the pour-plate technique, the combination of larger sampling numbers and fewer dilutions increases the accuracy of the tube-dilution method. However, its real advantages lie in convenience. The technique has four main advantages: (i) it is economical in use of reagents and apparatus; (ii) it is rapid and easy in operation because it relies on one sampling procedure and four transfers only; (iii) it is automatic in use in that it does not demand a decision as to which dilutions to plate and, in work on activity of antibacterials where the count can vary over a wide range, this may well be the greatest single advantage; (iv) the assessment of a count is simple, since the considerable effort of counting individual colonies is stored in the calibration series and can be used again and again.

There are two main disadvantages in the method as described in this paper. First, the lower end of the range is restricted by the size (0·1 ml.) of the sample taken to the

first tube. Obviously any count of less than 10/ml. is going to give less than 10^2 colonies in the tube used for matching, with consequent increase in sampling error. This factor was introduced deliberately, as the method is currently being used to assess the effect of synthetic antibacterial compounds on culture viability, and a large dilution is required to extinguish the effect of drug carried over to the counting medium. However, when there is no such obligation to dilute out toxic material, it is quite feasible to increase the sample to 1 ml. (decreasing BCM to 5 ml.) which improves the range tenfold.

The second disadvantage is imposed by the type of vessel used for the culture of the counting medium. This limits aerobiosis and prevents the method's application to strict aerobes. Nor has any effort been made to count strict anaerobes. Even with facultative anaerobes only a small number of species has been counted. These include some of the enteric bacteria (species of *Escherichia*, *Salmonella*, *Shigella*) and many strains of *Staphylococcus aureus* (unpublished results). There is probably little restriction on its use for facultative anaerobes, although some modifications in the medium might have to be made to accommodate the nutritional requirements of particular species.

The development of this method was begun when the author was working at Imperial Chemical Industries Ltd., Pharmaceuticals Division, Alderley Park, Macclesfield, Cheshire. The technical assistance of Mrs Glenda Crook is gratefully acknowledged. The correlation of the method with the standard plate count was verified in Glasgow by Mrs F. Mahmood.

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The Accumulation of Phenolic Acids in Tissue Culture Pathogen Combinations of *Solanum tuberosum* and *Phytophthora infestans*

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SUMMARY

Suspended tissue cultures of R 1-resistant *Solanum tuberosum* infected with race 4 sporangia of *Phytophthora infestans* developed toxicity in the culture fluid when tested after 10 days against a zoospore suspension of *P. infestans*. The toxic material was extracted into ether and salicylic, vanillic and *p*-hydroxybenzoic acids were identified in the toxic fraction by thin-layer chromatography, gas–liquid chromatography and mass spectrometry. Quantitative estimates of the amounts of material present in the toxic fraction allowed a mixture to be made which closely simulated the natural toxic material. Use of pure materials allowed an analysis of the behaviour of the zoospores with variation in dosage.

INTRODUCTION

Evidence for an 'immunizing' effect of pre-infection of cut slices of potato with an avirulent race of *Phytophthora infestans* was given by Müller & Börger (1940) from a study of the response of a series of potato varieties differing in susceptibility to *P. infestans*. Müller & Behr (1949) suggested that the resistance of the potato to *P. infestans* was the result of the production of substances, named by them 'phytoalexins', inimical to the invading fungus. The concept that resistance to attack by a wide spectrum of fungal pathogens and non-pathogens was engendered by the production of non-specific phytoalexins was further explored by Müller (1958) for *Phytophthora infestans* and *Monilinia fructigena* with *Phaseolus vulgaris* pods as host tissue, and by Cruickshank & Perrin (1965) for *Monilinia fructicola* and *Ascochyta pisi* with *Pisum sativum* as host. This and other work, summarized by Cruickshank (1963), led to the view that where a host actively resisted invasion by a pathogen a phytoalexin was produced as a response to fungal invasion. Such phytoalexins so far identified are relatively simple aromatic polycyclic compounds; work on the identification of phytoalexins has been confined to the products of plants acting as non-congenial hosts for a wide variety of fungi; some, e.g. pisatin, are also produced in response to tissue poisoning by compounds of heavy metals (Cruickshank & Perrin, 1965). Tomiyama *et al.* (1968) demonstrated and Katsui *et al.* (1968) characterized a new antifungal substance rishitin, which qualified as a phytoalexin, from tuber tissues of potatoes infected by an incompatible race of *P. infestans*. This suggested that the R gene-resistance system may also be based on the post-infectional production of phytoalexins.

Ingram & Robertson (1965) showed that tissue aggregates derived from tubers of *Solanum tuberosum* var. Orion (R 1) resisted the attack of *Phytophthora infestans* race 4. Ingram (1967) showed that, in contrast to suspended tissue cultures of cultivar Majestic, suspended tissue cultures of cultivar Orion inoculated with sporangia of *P. infestans* race 4 showed a subsequent development of toxicity such that the germination of test zoospores was totally prevented by the tissue-culture liquid 144 hr after initial infection. The nature of this toxic material is investigated below.

METHODS

Growth of tissue cultures. The methods used were in general as described by Ingram & Robertson (1965) and Ingram (1967). To increase the quantity of material for extraction Erlenmeyer flasks (1 l.) each containing 100 ml. of a dense 8-week *Solanum tuberosum* tissue suspension were inoculated with 10^6 sporangia of *Phytophthora infestans* and tested at intervals for the development of toxicity. Cultures of this size inoculated in this way generally showed marked toxicity at 10 days, as did parallel cultures prepared exactly as described by Ingram (1967). This was in contrast to the 6-day period for the full development of toxicity shown by Ingram. The same culture stock was used and there was a gradual increase in the time to development of full toxicity over the 3 years of this investigation. This increase was accompanied by a gradual change in the appearance of the cultures. These were at first dark brown and slow-growing and are now paler and grow faster. Ingram (1966) referred to a fast-growing white sector which developed in callus tissue cultures and which showed less marked resistance than the brown parent material. It appears likely that during the work reported here we have been selecting for faster growth of the suspended tissue cultures and there has been a parallel increase in the time required for the development of toxicity by the cultures. As soon as toxicity was noted the cultures were extracted. Control flasks of inoculated Majestic tissue cultures or uninoculated Orion tissue cultures, of the same age and cultural treatment, were harvested along with infected Orion cultures. Parallel experiments were done with tissue slices removed aseptically from tubers of Orion and Majestic.

Tests for toxicity. Qualitative tests for toxicity of extracts were made by inoculating 0·1 ml. of the material for assay with 1×10^4 freshly hatched zoospores on a clean sterile slide and estimating the degree of lysis or shrinkage of the zoospores in several fields after 10 min. Where quantitative estimates were required the method was modified and 0·5 ml. of solution at twice the required final concentration was mixed with 0·5 ml. of zoospore suspension adjusted to give 50,000 zoospores/ml. before dilution and 25,000 zoospores/ml. after mixing in the test solution. Then four drops of the mixture were placed on a slide and the percentage lysis of all zoospores in any 6 fields of the microscope, taken at random, was found after 3 hr. and, where required, the average germ-tube length after a 5 hr. period. Both methods of measurement gave similar estimates of the LD 50 dose of toxic material.

Chemical extraction of toxic material. At harvest, the tissue culture (usually 120 ml.) was filtered (suction) through acid-washed celite and the clear filtrate extracted with one-sixth of its volume of redistilled ether in a separating funnel. The extraction was repeated 6 times and the ether extracts (120 ml.) mixed. A 10 ml. portion of the mixed ether extract was evaporated under suction to dryness, the vessel flushed with nitrogen

and the residue taken up in 1 ml. distilled water before testing for toxicity. The aqueous solution remaining after extraction with ether was then evaporated in a rotary evaporator to remove remaining ether and a sample tested for toxicity against a standard zoospore suspension.

Gas chromatography. Before gas–liquid chromatography, an evaporated sample of the ether extract dissolved in pyridine was treated with hexamethyl disilazane (HMDS) in the presence of catalytic amounts of trimethyl chlorosilane (TMCS) to form volatile trimethyl silyl ethers and esters by the procedure of Dalgleish *et al.* (1966).

Derivatives of standard phenolic acids were prepared in a similar manner. Chromatographic separations were done by using a Perkin Elmer F.11 gas chromatograph fitted with a biomedical flame-ionization analyser, used in the single-column mode. Samples (usually 1 μl.) were injected into a 6 ft. $\times \frac{1}{4}$ in. (O.D.) glass column packed with 3% SE301 on AW-DCMS Chromosorb G. The temperature of the injection area was 270°, the oven temperature 175°; and the nitrogen carrier-gas flow-rate was 33 ml./min.

Thin-layer chromatography. MN Silica Gel G and silica gel/MN cellulose 300 plates, 20 cm. $\times$ 20 cm. glass, with the solvent system toluene + ethyl formate + formic acid 5 + 4 + 1 by vol. (Van Sumere, Wolf, Teuchy & Kint, 1965) were used for the analyses of extracts. Samples of phenolic acids dissolved in ether were used as standards. The plates were first examined under ultraviolet radiation (wavelength 365 nm.) and then sprayed with 2N-NaOH and again viewed by u.v. radiation to observe colour changes. The plates were then sprayed with diazotized *p*-nitroaniline and then with 5% (w/v) Na_2CO_3 (Van Sumere *et al.* 1965).

Ultraviolet spectrophotometry. Spectra were measured in 1 cm. optical cells using a Beckman DK-2A recording spectrophotometer.

RESULTS

Evidence for identification of compounds

The toxicity which developed in tissue cultures of potato Orion (R 1) when infected with *Phytophthora infestans* race 4 did not develop in tissue cultures of Majestic (rr) infected with race 4 over the same period of time nor did it appear in uninfected cultures of Orion.

The toxic fraction was removed by shaking the tissue culture filtrate with a variety of solvents immiscible with water (e.g. chloroform, butanol, ethyl acetate, ether); the best of these was ether because of the ease of recovery of the extracts at low temperatures and because, in contrast to the other solvents, re-distilled ether left no toxic residue after evaporation. Zoospore tests of the aqueous solution after extraction with re-distilled ether showed that all the detectable toxicity had been transferred to the ether. The ethereal solution was extracted either with 3% aqueous Na_2CO_3 or with 1 and 5% NaOH aqueous solution; the toxic fraction was recovered on acidification. At this point thin-layer chromatography showed the presence of one blue fluorescent and two deep purple (due to absorption) spots in the ethereal extract under u.v. radiation which gave colour reactions with diazotized compounds. The phenolic nature of at least one of the components of the extract was indicated by measurement of the difference spectrum between a portion of extract adjusted to pH 7 and another

adjusted to pH 12·3 which showed a peak at 298 nm., indicative of a non-conjugated phenolic hydroxyl group (Goldschmidt, 1954).

Mass-spectrographic examinations of a sample of the total ether extract of the infected Orion toxic material was made on an AEI MS902 instrument. Peaks were present which indicated compounds having mass numbers of 138 (corresponding to salicylic and *p*-hydroxybenzoic acids), 168 (corresponding to vanillic acid) and 178 (which might be a small amount of coniferaldehyde).

Gas–liquid chromatography of the trimethylsilylated extracts showed three well-separated peaks with retention times at 2·1, 3·2 and 5·2 min. These retention times corresponded with those for salicyclic acid, *p*-hydroxybenzoic acid and vanillic acid, respectively. Further gas chromatography of the Orion tissue culture toxic material and a mixture of vanillic, salicylic and *p*-hydroxybenzoic acid gave identical traces.

Thin-layer chromatography of the ether extract showed three main compounds which reacted with the diazotized *p*-nitroaniline spray and which had the same R_F values and colour reactions as salicylic, *p*-hydroxybenzoic and vanillic acids, respectively. The extract was then streaked on the plate and the areas of the plate corresponding to the sprayed marker spots were scraped off, eluted with ether and analysed individually by gas–liquid chromatography; each showed a single peak with a retention time corresponding to the standards, and the mass-spectrographic analysis of each gave the appropriate mass number (see Table 1). Concentrates of the eluted spots were toxic when tested against zoospores of *Phytophthora infestans*.

Table 1. *Toxic material from Phytophthora infestans: identity of bands separated by thin-layer chromatography and comparison of the properties of the bands with standard compound*

Band	R_F on TLC	Colour with diazo spray	Retention time of TMS derivative (min.)	Mass no.
1	0·46	Pink	3·5	138
2	0·51	Purple	5·4	168
3	0·61	Ochre	2·1	138
Salicylic acid	0·61	Ochre	2·1	138
p-Hydroxybenzoic acid	0·46	Pink	3·4	138
Vanillic acid	0·52	Purple	5·4	168

Quantitative estimations

Once the identity of the toxic compounds had been established it was of interest to determine the concentrations at which they were toxic and to look critically at the methods of assay. Müller (1958) tested a diluted series of suspected phytoalexins from Phaseolus and Pisum against newly hatched zoospores of *Phytophthora infestans* and noted that the normal response of the zoospores to high concentrations of phytoalexin was plasmoptysis within 60 sec., but that occasionally zoospores did not show plasmoptysis at lethal concentrations; in these cases shrinkage only took place. Müller used a dosage-response curve to define the LD50 dose and calculated the relative concentration in units derived from the reciprocal of the dilution fraction for the LD50 dose. Thus a dilution of 1/8 produced a mean germination rate of 50% and was taken to contain 8 units phytoalexin. When *Sclerotinia fructicola* was used for

assay the same calculations were made; but instead, with the LD 50 dose, a decrease to 50% of the mean germ-tube length was recorded. We used known amounts of pure salicylic, vanillic and *p*-hydroxybenzoic acid singly and together, and found that plasmoptysis invariably occurred in concentrations in excess of about 20 p.p.m., and that just below this concentration zoospores did not disintegrate but shrank and turned brown. These brown zoospores did not germinate, but some zoospores which did not shrink germinated and the numbers which germinated and the length of the germ tubes increased as the material underwent further dilution. The situation was further complicated by the effect of spore numbers. When zoospore numbers were large the effect of the toxic material was less than when the numbers were small.

It was of interest to discover what concentrations of toxic material were present in the infected tissue-culture liquor. Calibration of the peak area against concentration on an appropriate range of the gas chromatograph showed a straight-line relationship which could be used for quantitative estimates. When such estimates were made they varied between different sets of biological material; but the relative proportions of the three acids remained in the same order. In a typical experiment harvested on 9 January 1968 the pooled contents of 12 flasks of Orion tissue infected by *Phytophthora infestans* on 21 December 1967 were calculated to contain 12·85 p.p.m. total phenolic acids in the aqueous layer. These were in the proportions salicylic:vanillic:*p*-hydroxybenzoic acid :: 111:46:27. Since a single flask yielded 7·78 g. fresh wt tissue, the approximate yield of toxic material was 47 μg./g. fresh cell wt.

To verify this estimation an artificial mixture in the proportions given above was made up (mixture Z) and tested on the same day in a dilution series in parallel with the original Orion extract; the results showed that the natural toxic material had an LD 50 dose of 12·5 p.p.m., while that of the mixture was 25 p.p.m. This suggested that the extract of Orion tissue culture might have contained small quantities of other materials which increased its toxicity. Thin-layer chromatography plates did show traces of other materials which were not characterized on these plates or by gas chromatography. There was also a degree of variability in the test zoospores. The LD 50 dose for mixture Z was determined on nine different occasions and varied from 16·5 to 34·5 p.p.m.

It remains to be asked whether the extraction of phenolic acids from the mixture and the tests for toxicity in aqueous solution did not artificially exaggerate the part played by these substances. Mixture Z, containing a total of 185 p.p.m. in water, was at pH 3·3. Tests on zoospores with phosphate and acetate buffers showed that the buffers themselves caused lysis at all pH values. Very dilute solutions of HCl allowed us to examine a range from pH 2 to pH 4·5. The zoospores of *Phytophthora infestans* germinated well at pH 3·7; above pH 3·3 the zoospores lysed. However, mixture Z at a dilution giving 18·5 p.p.m., which was the value normally found in experiments, was at pH 4·3, suggesting that the pH value of the phenolic acids, at the concentrations normally tested, was not responsible for the zoospore inhibition. The reverse situation when, for example, salicylic acid was added to fresh tissue culture medium (pH 5·6) showed that the concentration of the acid had to be raised to 200 p.p.m. (4·85) to bring about total zoospore lysis. When tissue culture medium in which potato cells of cultivar Majestic had grown for 4 weeks (pH 5·8) was used, lysis and inhibition were obtained at 100 p.p.m. salicylic acid added (pH 4·1). Finally, when sodium salicylate was tested against zoospores it inhibited germination at 25 p.p.m., but not at 12·5 p.p.m.

In this work we did not observe any marked difference in the degrees of toxicity of the three acids or any evidence of synergistic effects of the mixture.

DISCUSSION

It is well understood that there is a change in the metabolism of infected resistant tissues of plants in the direction of the accumulation of phenolic substances (Farkas & Kiraly, 1962). There are certain difficulties in interpretation of results, however, for attempted invasion is accompanied to a greater or lesser extent by death of the invaded tissues. At one extreme a single cell will undergo necrosis following invasion and the invading hypha will not be able to grow further, e.g. the invasion of the cells of R gene-resistant potatoes by *Phytophthora infestans*. At the other extreme, invasion of a tuber will proceed for a considerable distance before death of the tissue takes place. It has thus been difficult to determine how far the phenolic materials produced are a response to invasion by the fungus and how far they are a consequence of the death of the plant cells. Müller's approach to the problem (1956, 1958) was to compare the materials secreted into the infection drops over a short period of time when inoculum, in water, was placed on exactly comparable resistant and susceptible varieties and the infection drop was then assayed for toxicity at intervals against freshly hatched zoospores of *Phtophthora infestans*. Although Müller's work began with *P. infestans* and potato, he found it convenient to develop his investigations with pods of *Phaseolus vulgaris* as recipients of massive infective doses of *P. infestans* and *Monilinia fructicola*. In the investigation reported here tissue culture suspensions were used because Ingram had shown that they reacted in a comparable manner to whole plants and because the liquid in which the tissues and infecting spores were suspended acted as a very large 'infection drop' and allowed the accumulation of reasonably large amounts of any inhibitory substances. Also, there was a marked difference between freely growing tissue cultures and freshly cut potato tuber slices, which tended to show the production of phenols (Tomiyama, 1963) as a wound reaction. Against this, it must be made clear that tissue-culture cell walls of potato have greater amounts of lignin than normal cells and that *P. infestans* can grown to a limited extent in the tissue culture medium with consequent metabolic effects. However, it is felt that the demonstration by Ingram that toxic substance(s) accumulate in infected suspended tissue cultures of cultivar Orion (R 1 resistant) while it fails to accumulate in uninfected suspended tissue cultures of cultivar Orion or infected suspended tissue cultures of cultivar Majestic, is reasonable ground for suggesting that the toxic material might be associated with the resistance reaction and might in fact be a 'phytoalexin'.

Investigation of the toxic tissue-culture media has shown the consistent presence of three simple benzoic acids, namely *p*-hydroxybenzoic, vanillic and salicylic acids, as the chief constituents. Moreover, during the fractionation procedure, toxicity increased step by step with procedures which eventually led to the isolation of these acids, and a mixture of the acids in the proportions of and at concentrations comparable to that found in the toxic tissue-culture fluid did closely parallel the natural toxin when compared in a dilution series, and gave a similar value for the LD 50 dose.

It remains to be discovered whether these phenolic acids produced in this situation are of significance only in this situation, or whether they have a wider significance in the resistance of plants. Tomiyana *et al.* (1968) implicated as the potato phytoalexin

a terpenoid, rishitin, which we did not find. From its structure it should be much less water-soluble than the benzoic acids and should not accumulate in the tissue culture medium. We have yet to test the tissue-culture cells for the accumulation of post-infection toxic material. On the other hand, Fawcett & Spencer (1967) showed a post-infection accumulation of benzoic acids in resistant apple varieties infected with brown rot fungus. Since lignification of cell walls naturally follows infection of the potato (Knee, 1967, personal communication) and since the pathway to the benzoic acids would be *via* cinnamic acid, which also takes part in the synthesis of lignin (Pridham, 1965), we may have been dealing with a special modification of the lignification process in the resistant potato tissue culture. This modification would involve either the induction or activation of the β-oxidation pathway leading from cinnamic acids to benzoic acids. Until the relative amounts of lignin and benzoic acids formed can be estimated it is difficult to suggest whether or not benzoic acids are being formed at the expense of lignin or in addition to lignin. Experiments to investigate the change in metabolic pathways involving the cinnamic acids are at present in progress.

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With 1 *plate*
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The Chemical Composition of Isolated Cell Walls of *Cyanidium caldarium*

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SUMMARY

The acid-resistant and heat-resistant alga *Cyanidium caldarium* yields cell-wall preparations which are unusually rich in protein (50 to 55%) and contain only small amounts of polysaccharides (hemicellulose, 12 to 14%; cellulose, 3 to 4%). At least 13 amino acids are present in the cell walls, but diaminopimelic acid, muramic acid and amino sugars are absent. It is suggested that Cyanidium is more closely related to the green rather than the blue-green algae.

INTRODUCTION

The remarkably acid-tolerant and heat-tolerant unicellular alga *Cyanidium caldarium*, which will grow at pH 2·0 in sulphuric acid and at temperatures up to 80°, is a regular member of the microflora in acid hot springs throughout the world. Its taxonomic position is still undetermined and it possesses characteristics of blue-green as well as of green algae. The mode of reproduction by the formation of autospores is similar to that of the green alga Chlorella (Allen, 1959) and all of the organelles, except the pyrenoid, found in Chlorella can also be detected in Cyanidium cells (Rosen & Siegesmund, 1961; Mercer, Bogorad & Mullens, 1963; Staehelin, 1968). In contrast, in Cyanidium the chromoprotein C-phycocyanin is spectroscopically indistinguishable from that of the blue-green alga *Microcoleus rupicola* and, of the chlorophylls, only chlorophyll-a is present (Allen, 1959). Staehelin (1968) was able to show with the freeze-etching technique that Cyanidium cells, during their life cycle, also develop unusual surface differentiations which are not found on the plasmalemma membranes of either Chlorella or blue-green algae. Apart from a reported absence of diaminopimelic acid (Work & Dewey, 1953), the composition of Cyanidium cell walls does not appear to have been investigated. The present work was undertaken to see whether determinations of cell-wall composition of *C. caldarium* would help to explain its systematic position, and its tolerance to acid and heat.

METHODS

Organism. The strain of *Cyanidium caldarium* isolated by Allen (1959) was obtained from the Cambridge algal collection (Botany School, Cambridge).

Cultures. The organism was grown in the inorganic medium (pH 2·8) of Allen (1959) with glucose (10 g./l.) added aseptically to the sterilized medium. Volumes (1 l.) of medium in 2 l. conical flasks were inoculated and incubated in an enclosed gyratory shaker (Gallenkamp Ltd.) at 45° and 200 rev./min. for 7 days under continuous illumination (100 W electric light, 18 in. above shaker top window) with occasional (2 to 3 times/day) gassing with CO_2. During this time a heavy growth of organism, initially yellowish and finally dark green, was obtained; the cultures reached finally pH 1·8 to 1·9. The organisms were allowed to settle under gravity and most of the liquid decanted; the more concentrated suspension was then centrifuged (1000 *g*) after which the deposited organisms were washed by suspension in water and then recentrifuged down.

Preparation of cell walls. Wet organisms, diluted to a thin paste with water, were mixed with an equal weight of Ballotini beads (no. 13) and disrupted by blending for 4 × 10 min. at full speed in a Lourdes Omnimix homogenizer with the blending-pot immersed in an ice bath. The blended mixture was diluted with water and the suspension of disrupted organisms decanted from the beads, which were then washed free from residual cell debris. Any undisrupted organisms and beads were removed from the combined suspensions by centrifugation at 1000 *g* for 2 to 15 min. after which the walls were harvested by centrifuging for 10 min. at 4000 *g*. The initial crude wall preparation was washed 3 to 4 times by suspension in water and centrifugation until the aqueous supernatant fluid was clear and colourless. Finally the grey wall preparation was washed by suspension for 5 min. and centrifugation, twice from M-NaCl and then twice from water to give preparation A. One portion of wet preparation A was washed by a similar treatment once in 0·5% sodium lauryl sulphate solution for 5 min. followed by two washings with water to give preparation B; a second portion of preparation A was similarly washed 4 times with the sodium lauryl sulphate solution and twice with water to give preparation C. All these wall preparations were freeze-dried.

Microscopical examination of the wall preparations. The wall preparations were examined by the optical and electron microscopes. Sectioned material for the electron microscope was prepared by fixing the walls in 2% $KMnO_4$ in tap water for 30 min., after which they were washed with water, dehydrated with ethanol and finally embedded in methacrylate (10 parts butyl-, 3 parts methyl-methacrylate, with 3% dichlorobenzoyl-peroxide (Luperco CDB; Lucidal Div., Novadel-Agene Corp., Buffalo, N.Y.) as accelerator) which was polymerized overnight at 60°. Unsectioned walls were shadowed with U_3O_8 after allowing a dilute suspension of walls to dry in air on Formvar-carbon-coated grids. Freeze-etch replicas for electron microscopy were also prepared by the technique of Moor & Mühlethaler (1963), but it was difficult to assess the purity of isolated wall fractions by this method.

Analyses of walls. Moisture and ash contents of the walls were measured by heating, respectively, at 100° and 500°.

Total nitrogen was measured by the micro-Kjeldahl procedure of Bathurst & Mitchell (1958). Total protein was measured by the method of Lowry, Rosebrough,

Farr & Randall (1951) as modified for insoluble protein. Total crude lipids were measured by extracting the walls by shaking at room temperature with chloroform + methanol (3 + 1 by vol.) for several hours. The combined extracts were evaporated to dryness and the residue weighed.

Carbohydrates were analysed by a variety of methods. Water-soluble polysaccharide and pectic substances in the walls were extracted by successive treatments for 15 min. with boiling water (25 ml.) and for 2 hr with boiling ammonium oxalate (0·5%, 50 ml.). Polymer hexoses and uronic acids were measured with anthrone and carbazole reagents, respectively (Bailey, 1967). Hemicelluloses and cellulose were measured by differential acid hydrolysis. Portions (20 mg.) of wall were hydrolysed for 2 hr. at 100° in N-H_2SO_4 and centrifuged to give a supernatant fluid (containing hemicellulose sugars) and a deposit which was dried with acetone. The deposit material was treated with 72% H_2SO_4 (0·2 ml.) for 4 hr at room temperature, diluted with water (4 ml.) and heated at 100° for 2 hr, to yield a second hydrolysate (containing cellulose sugars). Reducing sugars in the hydrolysates were measured by the microcuprimetric method of Nelson (1944) and calculated as anhydro-xylose and anhydro-glucose for hemicellulose and cellulose, respectively. Portions of the hydrolysates were neutralized with solid $BaCO_3$, filtered and concentrated for paper chromatographic analysis.

The nature of the possible hemicellulose and cellulose polymers was further examined by their solubilities in alkali. A portion (50 mg.) of wall was extracted by shaking overnight at room temperature with 2 × 10 ml. of aqueous KOH (24% w/v) and centrifuged, after which the alkali-insoluble residue was hydrolysed by treatment with 72% H_2SO_4. The alkaline solution was acidified with acetic acid and ethanol (2 vol.) added, to give a precipitate which was hydrolysed in N-H_2SO_4 for 2 hr at 100°. The acid hydrolysates were treated with $BaCO_3$ and prepared for paper chromatography as already described.

Chromatograms for monosaccharide analyses were developed with ethyl acetate + water + pyridine (2 + 2 + 1, by vol.) or ethyl acetate + acetic acid + formic acid + water (9 + 1·5 + 0·5 + 2, by vol.) and sugars located with aniline hydrogen phosphate spray reagent. Quantitative chromatograms used the method of Wilson (1959) and the same aniline hydrogen phosphate spray reagent when the papers were sprayed and heated under carefully controlled conditions which gave good recoveries (over-all error ± 5%) of sugars. Amino sugars in the amino acid hydrolysate were located on chromatograms developed either with the solvent systems used for sugar analysis, or with those used for amino acid analysis, with the following spray reagents: ninhydrin + collidine, *p*-dimethylaminobenzaldehyde (Partridge, 1948; Crumpton, 1959) and thiobarbiturate (Warren, 1960).

For amino acid analysis hydrolysates were prepared by heating the walls (20 mg.) at 100° for 18 hr in 6 N-HCl (2 ml.) in a sealed tube and then removing the acid by repeated evaporation in vacuum over solid NaOH. Amino acids were identified on chromatograms developed in phenol-water, followed by *n*-butanol + acetic acid + water (60 + 15 + 25, by vol.), and in ionophoretograms run in acetic acid + formic buffer (pH 2·0, Smith, 1960) and sprayed with ninhydrin + collidine.

RESULTS

The yield of wet organisms from 16 l. of culture was 266 g., containing 13·5% of freeze-dried solid. From 160 g. of these wet organisms (21·6 g. freeze-dried solid) 1·82 g. freeze-dried preparation A walls was obtained (1·64 g. corrected for moisture). All of the freeze-dried wall preparations contained about 10% moisture and all results given below are on a moisture-free basis.

According to Punnett & Derrenbacker (1966) prolonged washing with sodium lauryl sulphate is necessary to remove gross contamination of cytoplasmic protein from many algal wall preparations. When a portion of preparation A was submitted to this type of prolonged washing procedure to give preparation C, the total nitrogen removed in the combined washings (0·59 mg.) represented only 4·5% of the total nitrogen (12·9 mg.) in the initial preparation A, suggesting that only a small amount of preparation A had actually been solubilized.

Microscopic examination of wall preparations

Whole *Cyanidium caldarium* organisms are easily distinguished from isolated walls in the light microscope. In the wall preparations used for chemical analysis only very few intact organisms were detected. Micrographs of a sectioned permanganate-fixed pellet of preparation A (Pl. 1, fig. 1, 2) showed the walls to be strongly stained and revealed no layering, while the small amount of cytoplasmic debris still adhering to the walls showed as dark granular material (Pl. 1, fig. 2). A similarly prepared micrograph of preparation B (Pl. 1, fig. 3) showed that the single sodium lauryl sulphate washing had nearly completely removed the cytoplasmic contamination without affecting the walls. The micrographs of preparation C (Pl. 1, fig. 4), however, showed that the prolonged washing with sodium lauryl sulphate resulted in a partial disintegration of the walls into smaller fragments. After heavy-metal shadowing electron microscopy of air-dried wall preparation A showed the walls to have a fine granular structure (Pl. 1, fig. 5, 6). Several attempts to detect fibrils, by electron microscopy, after removing some of the matrix from the walls by various treatments, both separately and sequentially with potassium hydroxide (4%, w/v), hydrochloric acid (0·25 M) and concentrated hydrogen peroxide + glacial acetic acid mixture (1 + 1, by vol.) met with no success, probably because of the very small amount of cellulose present. In these attempts unstructured masses of sponge-like material were formed or the walls disintegrated completely. The absence of highly ordered material such as fibrils in the walls of Cyanidium was confirmed with X-ray diagrams of untreated isolated walls. The diagrams indicated the presence of a keratin-like substance, but the lines were always very weak and diffuse.

Composition of the walls of Cyanidium caldarium

Results from the analyses of the wall preparations are listed in Table 1. Qualitative and quantitative paper chromatograms of the hemicellulose hydrolysates from the analyses showed spots corresponding to galactose, glucose, mannose and xylose in the ratio of 5·2:4·5:2·5:1·0, but only traces of arabinose and uronic acids and no methylpentoses. The cellulose hydrolysates showed only glucose on the chromatograms. After prolonged extraction of a portion of wall preparation A with 24% KOH the alkali-insoluble residue gave a hydrolysate showing only glucose on the chromato-

grams. Hydrolysates of the alkali-soluble polysaccharide showed on the chromatograms all of the expected hemicellulose sugars, with evidence, not further examined, that much of the galactose was present in a more soluble polymer than were the glucose, mannose and xylose.

Table 1. *Composition of Cyanidium caldarium cell walls*

All results are calculated as % of moisture-free cell walls.

	Wall preparation		
	A	B	C
Ash	10·0	—	5·8
Total nitrogen	8·96	8·55	9·11
Total lipids	2·0	—	—
Hot-water-soluble polysaccharide	0·67	—	—
Oxalate-soluble polyuronide (pectin)	0·035	—	—
Hemicellulose	13·73	15·00	14·50
Cellulose	3·83	2·74	4·11

Table 2. *Amino-acid composition of Cyanidium caldarium cell walls*

Amino acids detected with ninhydrin+collidine on paper chromatograms of 6 N-HCl hydrolysates. Intensities of spots judged visually and graded as very intense, ++++; moderately intense, +++; weak intensity but definite, ++; faint, +; absent, −.

	Wall preparation		
Amino acid	A	B	C
Glutamic acid	++++	++++	++++
Serine	++++	++++	++++
Threonine	+++	+++	+++
Alanine	+++	+++	+++
Glycine	+++	+++	+++
Tyrosine	+++	+++	+++
Valine	+++	+++	+++
Leucine-isoleucine	++	++	++
Aspartic acid	++	++	++
Proline	++	++	++
Lysine	++	++	++
Methionine	+	+	+
Histidine	+	+	+
Hydroxyproline	+	−	−
Phenylalanine	+	−	−
Arginine	+	−	−
Unknown blue spots			
X1	+++	+++	+++
X2	++	+	−
X3	+	−	−
X4	++	+	−

The amino acid composition of the three wall preparations is listed in Table 2. Cysteine and diaminopimelic acid were not detected in the hydrolysates. Unknown X1, present in significant amounts, moved on the chromatograms in almost the same way as diaminopimelic acid, although its stain colour with collidine+ninhydrin was slightly different. Ionophoresis at pH 2·0 of wall hydrolysate with and without added authentic α,ε-diaminopimelic acid clearly showed none of this acid in the hydrolysates and that the unknown X1 was different. There was no sign of components

corresponding to glucosamine, galactosamine or muramic acid on chromatograms of any of the wall preparation hydrolysates.

The wall preparations could not be dissolved in N-alkali in sufficient concentration for the biuret measurement of total protein. With the Folin reagent an alkaline solution gave an intense blue colour, corresponding in the case of preparation C to 65% protein (bovine serum albumin as standard).

DISCUSSION

The wall carbohydrates of several green algae have been examined and a mixture of polysaccharides composed principally of galactose, mannose, xylose and glucose appears to be fairly common (Kreger, 1962). Northcote, Goulding & Horne (1958) found that the walls of Chlorella were rich in hemicellulose polymers (30%) composed of galactose, glucose, mannose, xylose and arabinose, and cellulose (16%). Although a mixture of carbohydrates composed of the same monosaccharides was found in the Cyanidium walls the amounts were very much lower and, in the case of cellulose, very low indeed. The walls of fewer examples of blue-green algae have been examined chemically (Echlin & Morris, 1965), and these were found to contain mucopeptide with amino sugar and muramic acid rather than simple polysaccharides. Neither glucosamine nor muramic acid was found in the Cyanidium cell wall hydrolysates. Northcote *et al.* (1958) and Punnett & Derrenbacker (1966) detected glucosamine in Chlorella walls.

The presence of a large variety of amino acids in the wall protein component of Cyanidium is in line with findings for Chlorella (Northcote *et al.* 1958), Nitella and Chaetomorpha (Thompson & Preston, 1967) and higher plants (Lamport, 1965). Diaminopimelic acid, typically present in bacterial and blue-green algal walls (Echlin & Morris, 1965), was clearly absent from the wall hydrolysates of Cyanidium, a finding in agreement with the results of Work & Dewey (1953) for whole Cyanidium organisms.

The present results tend to support the suggestion that *Cyanidium caldarium* occupies an intermediate position between the blue-green and the green algae, with possibly a closer relationship to the green than to the blue-green algae.

The most outstanding feature to emerge from the present work with *Cyanidium caldarium* walls is the high (50 to 55%) protein content. There is of course the possibility that some of this protein is cytoplasmic protein which has become bound to the cell walls during their isolation. This seems unlikely in view of the limited spectrum of amino acids found in the hydrolysates and the evidence for the removal of cytoplasmic debris by washing (Table 1 and Pl. 1, fig. 2, 3). As far as we know, no other plant or algal walls have been reported with such a high percentage of protein. Typical results are *Chlorella pyrenoidosa*, 7 to 9% (Northcote *et al.* 1958); Nitella, 8 to 10%, Chaetomorpha, 7 to 9% (Thompson & Preston, 1967); and Sycamore cell cultures, 7·5 to 11% (Lamport, 1965). All electron microscope preparations of the Cyanidium walls indicate that the material is very dense and it seems possible that the closely packed proteins could provide the cells with more than just mechanical protection. Perhaps the high protein content of the walls is related to the heat and acid tolerance of the organisms.

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EXPLANATION OF PLATE

Fig. 1. Section of isolated walls of *Cyanidium caldarium* washed with water and M-NaCl (preparation A), fixed with 2% $KMnO_4$ and embedded in methacrylate. × 16,000.

Fig. 2. Higher magnification of the same preparation as in fig. 1. The granular material adhering to the cell wall surfaces is probably due to cytoplasmic contamination. × 30,000.

Fig. 3. Sectioned walls prepared as described for fig. 1 but additionally washed with 0·5% Na laurylsulphate for 5 min. (preparation B). This treatment removed nearly all the granular material from the wall surfaces. × 30,000.

Fig. 4. Sectioned walls prepared as in fig. 1 but treated additionally for 4 × 5 min. with 0·5% Na laurylsulphate (preparation C). The walls show signs of disintegration. × 30,000.

Fig. 5. Isolated wall of *C. caldarium* washed with water and M-NaCl (preparation A) and shadowed with U_3O_8. × 22,000.

Fig. 6. Higher magnification of the same preparation as Fig. 5. The wall material appears finely granular; no fibrils detectable. × 40,000.


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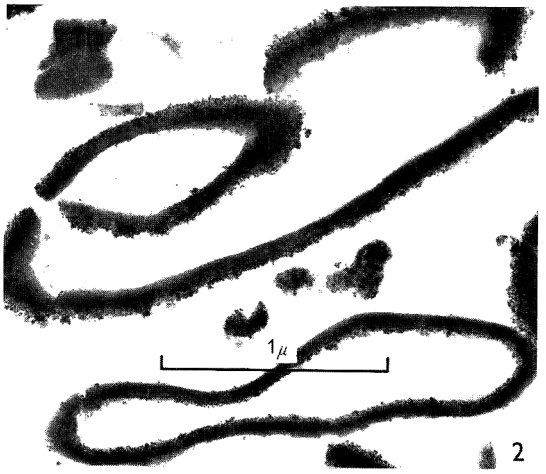
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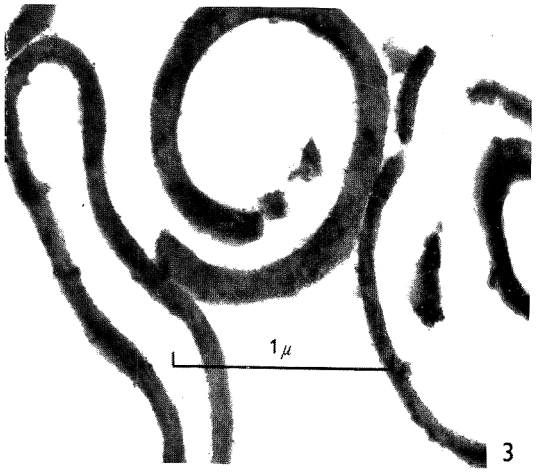
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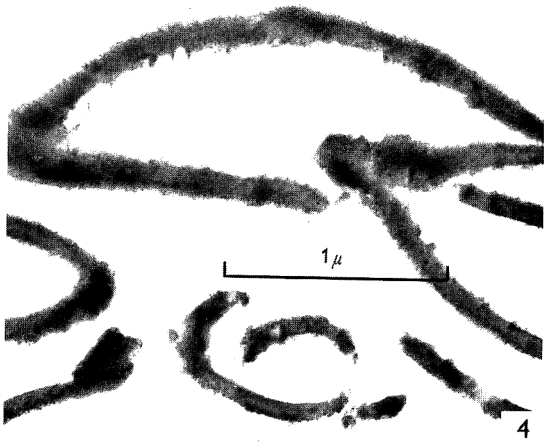
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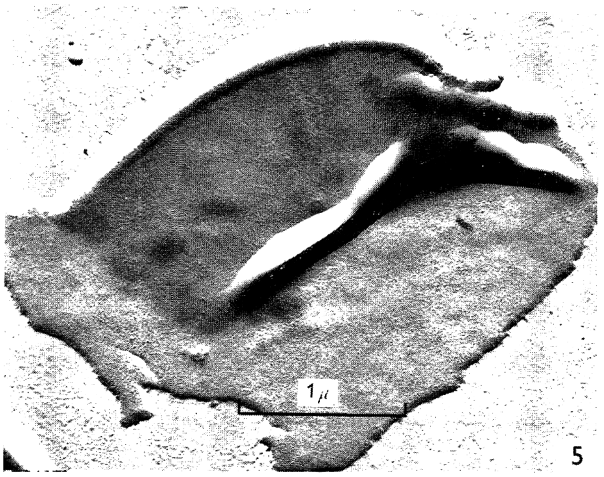
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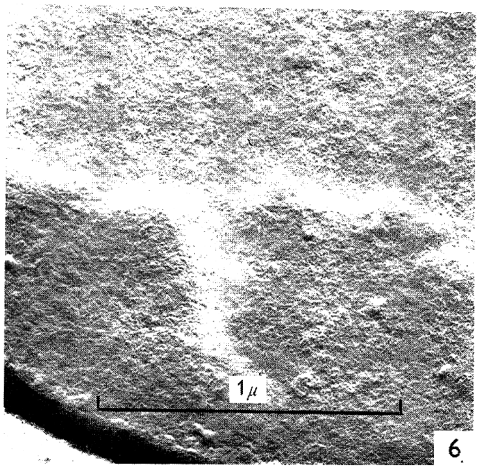
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Deferred Metabolism of Glucose by *Clostridium tetanomorphum*

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SUMMARY

Clostridium tetanomorphum grew in medium containing yeast extract when supplied with glutamate, histidine, glucose, maltose or pyruvate. Measurements of Q_{H_2} values and specific activities for two enzymes, phosphofructokinase and β-methylaspartase indicated that the glucose and glutamate fermentations were inducible. With glucose, the onset of active glucose metabolism was delayed in preference for energy sources provided by yeast extract. The inhibitory mechanism which was responsible for this delay did not appear to be a typical enzyme repression but rather a catabolite inhibition. No significant changes in the cobalamin content of the organisms accompanied growth with different substrates.

INTRODUCTION

In a study of hydrogen evolution by anaerobes, washed suspensions of *Clostridium tetanomorphum* have been shown to ferment several amino acids and glucose, maltose, glycerol, pyruvate, fumarate, malate (Woods & Clifton, 1938). The organism was used by Barker (1956) to elucidate the path of glutamate fermentation. This led to the discovery of the cobamide coenzymes which are synthesized by this organism, and which are essential coenzymes for glutamate mutase, the first enzyme in the glutamate fermentation (Barker, Weissbach & Smyth, 1958). The object of the present work was to find some substrate other than glutamate which would serve as a major energy source, in order to study possible mechanisms of metabolic regulation in this strict anaerobe.

METHODS

The organism. Clostridium tetanomorphum (ATCC 3606) was maintained on slopes of blood agar (Oxoid) containing 7% horse blood and 0·1 M-sodium L-glutamate. After growth at 37° for 24 hr sealed with an alkaline pyrogallol plug, the cultures were stored at 2° and remained viable for at least a month.

Media and growth of organisms. The salts+yeast extract basal medium of Barker, Smyth, Wilson & Weissbach (1959) was modified by adding NH_4Cl (0·02 M) and omitting sodium hydrosulphite; substrates (0·1 M) were added after separate sterilization (121° for 15 min.). For the growth of small volumes (10 ml.) ⊥-tubes with alkaline pyrogallol plugs, sealed with bungs, were used; such tubes facilitated the measurement of growth when the organisms were beginning to sediment. The inoculum was 1% (v/v) of a late exponential phase culture.

Two types of vessel were used for growth of the organism:

(1) For volumes of up to 200 ml., a 250 ml. Erlenmeyer flask with a 15 × 125 mm.

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test-tube welded at the base was used. The flask was sealed with an assembly which allowed tipping into the tube for measurement of growth and at the same time maintained an anaerobic atmosphere but prevented a build-up of gas pressure. This assembly consisted of another test-tube with a small hole at one side of the closed end; this was passed through a rubber bung and plugged with cottonwool. The flask + medium and the bung assembly were sterilized separately, appropriate substrates added, the medium inoculated and the bung put into position. The air space was evacuated, flushed with $H_2 + CO_2$ (95 + 5, v/v) and a plug soaked in alkaline pyrogallol was placed in the tube which was finally sealed with a bung fitted with a Bunsen valve (a piece of rubber tubing split diagonally across its width and closed with a glass rod at the distal end).

(2) When sampling was also required a flask, as described above (or larger), was fitted with an exit-tube which could be closed with silicone tubing and a screw clip. The neck of the flask was fitted with a bung which had several plugged tubes passing through it. One passed to the bottom of the flask for gassing with $H_2 + CO_2$ (95 + 5, v/v), a second was used to evacuate the gas space and a third passed to a Bunsen valve as described above. The freshly autoclaved medium was inoculated, the bung put into position and after evacuating, gas ($H_2 + CO_2$, 95 + 5, v/v) was bubbled through the medium for 10 min. During incubation the bunsen valve was left open; for sampling it was closed with a screw clip, a positive pressure of gas ($H_2 + CO_2$) was introduced and a sample collected in an ice-cooled receptacle from the exit at the base of the flask. Because of the risks of contamination entailed by this procedure all cultures were tested for purity by streaking samples on blood agar (Oxoid) + glucose (0·1 M) during each experiment. The samples withdrawn from the culture were cooled rapidly, the organisms sedimented by centrifuging (5000 *g* for 10 min.) and the supernatant fluid retained for assay of substrates remaining in the medium.

Growth was assessed turbidimetrically with an EEL colorimeter (Evans Electroselenium Ltd.), with a green filter no. 624 and expressed as mg. dry wt./ml., calculated from a standard curve.

Assay of pyruvate, glucose and glutamate. These compounds were assayed enzymically in dilutions of the culture supernatant fluids: pyruvate by the method of Bücher, Czok, Lamprecht & Latko (1963), glucose by the method of Bergmeyer & Bernt (1963) and glutamate according to Wyngaarden & Ashton (1959).

Experiments with washed-cell suspensions. The fermentation of glucose, glutamate and pyruvate by suspensions of washed organisms was measured manometrically by using standard techniques. Organisms were harvested by centrifuging at 10,000 *g* at 0° for 10 min. washed twice in phosphate buffer (0·05 M, pH 7·4) and resuspended in the same buffer. Hydrogen production by organisms (equiv. 10–20 mg. dry wt) was measured in phosphate buffer (0·05 M, pH 7·0) with substrate (0·05 M) in an atmosphere of H_2 and with KOH (10 %, w/v) in the centre well.

Assay of enzymic activities. Cell-free extracts were prepared from washed organisms suspended in phosphate buffer (0·02 M, pH 7·4) at a concentration equivalent to 300 mg. wet wt/ml. by disruption with an M.S.E. ultrasonic disintegrator for 20 sec. at 2°. The supernatant fluid obtained after centrifuging at 15,000 *g* for 20 min. at 0° was used for assaying phosphofructokinase by the method of Opie & Newsholme (1967). The assay for β-methylaspartase was that of Barker *et al.* (1959) with the exception that tris buffer (0·1 M, pH 9·0) was used. Protein was estimated by the method of Lowry,

Rosebrough, Farr & Randall (1951). Specific activities are expressed in terms of μmoles substrate changed/min./mg. protein.

Assay of cobalamin. Cobalamin was assayed microbiologically with *Euglena gracilis* var. *bacillaris* and *Escherichia coli* strain 113/3 (a cobalamin or methionine-requiring auxotroph; Davis & Mingioli, 1950) using the methods described by Foster, Jones & Woods (1961). *Clostridium tetanomorphum*, harvested from 200 ml. medium when approaching maximum growth, was washed once with an equal volume of distilled water and resuspended in 10 ml. potassium phosphate buffer (0·02 M, pH 6·5) containing KCN (0·01 M). Samples of this were used for protein estimations and for cobalamin assay. For the latter, samples (3 ml.) were autoclaved at 115° for 10 min., cooled and digested with Seitz-filtered papain (0·3 mg./ml. final concn.) at 25° for 48 hr. The samples were then adjusted to pH 4·5 with acetic acid, heated to 100° for 10 min., and denatured protein removed by centrifuging. The supernatant fluid was used for the assay of cobalamin with cyanocobalamin (vitamin B_{12}) as standard. Initially the medium contained 18 mμg. vitamin B_{12}/ml. for Euglena assay and after growth the cultures contained approximately 45 mμg. vitamin B_{12}/ml., more than 80% of which was recovered in the washed organisms.

Materials. The enzymes required for glucose, glutamate and pyruvate assays were purchased from C. F. Boehringer und Soehne G.m.b.H. Mannheim, Germany, as were NAD, $NADH_2$, ATP and glucose-6-phosphate. Twice-crystallized papain and cyanocobalamin were from British Drug Houses Ltd., Poole, Dorset. The sources of other reagents were: 3-acetylpyridine NAD (Calbiochem, Los Angeles, Calif., U.S.A.), DL-citramalic acid (Aldrich, Milwaukee, Wisconsin, U.S.A.), mesaconic acid (Fluka A.-G., Buchs SG., Switzerland), β-methyl-DL-aspartate (Sigma Chemical Co., St Louis, Ma., U.S.A.) and yeast extract (Difco Laboratories Inc., Detroit 1, Michigan, U.S.A.).

RESULTS

Growth experiments

When *Clostridium tetanomorphum* was incubated in media containing yeast extract (0·3%) growth occurred with the following substrates: glucose, maltose, L-glutamate, L-histidine and to a lesser extent with pyruvate. No growth was observed with the yeast extract alone, nor with added fructose, L-aspartate, L-threonine, α-ketoglutarate, succinate, fumarate or L-malate, some of which were originally shown to be fermented by washed suspensions of the organism (Woods & Clifton, 1938). β-Methyl-DL-aspartate, mesaconate and DL-citramalate, intermediates in the fermentation of glutamate, were also unable to support the growth of the organism in this medium. With glutamate (0·1 M) as substrate maximum growth was obtained when the concentration of yeast extract was increased to about 1·5% (Fig. 1) and this concentration was used in all subsequent experiments. Even at this concentration of yeast extract, growth in the absence of glutamate was less than 5% of the amount reached in its presence.

Growth of *Clostridium tetanomorphum* with glutamate, glucose or pyruvate became linear after a short exponential phase. The mean generation times calculated from these short phases were: for glutamate, 1·2 hr; glucose 1·5 hr; pyruvate, 1·2 hr. Measurements of growth yields were made to ensure that glucose and pyruvate were serving as principal energy sources (Fig. 2). In the region, where the yields were pro-

portional to substrate concentration, no substrate remained in the medium after growth and the yield coefficients calculated were 41·0 g. dry wt organism/mole of glucose consumed, and 6·0 and 8·0 for glutamate and pyruvate, respectively. Assuming that Y_{ATP} is 10 then the corresponding ATP yields, 4·1 for glucose, 0·6 for glutamate and 0·8 for pyruvate, are consistent with the anticipated yields of 0·5 to 1·0 mole ATP/mole glutamate or pyruvate converted to butyrate or acetate, respectively, by the recognized metabolic routes, plus an extra 2 moles ATP/mole of glucose converted to pyruvate via the glycolytic pathway.

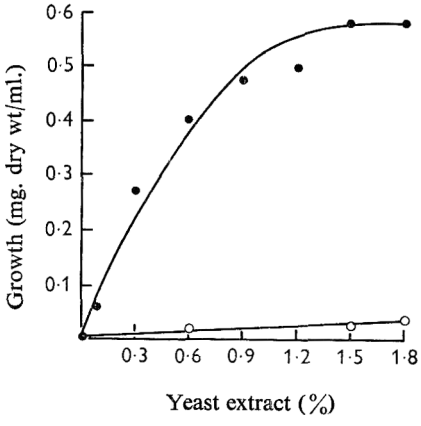

Fig. 1

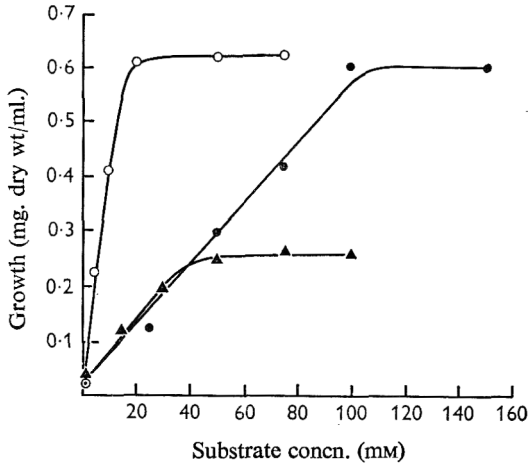

Fig. 2

Fig. 1. The effect of yeast extract concentration (%, w/v) on the growth of *Clostridium tetanomorphum*. ●, With glutamate (0·1 M); ○, without glutamate.

Fig. 2. Growth as a function of substrate concentration. The medium contained yeast extract (1·5 %). ●, Glutamate; ○, glucose; ▲, pyruvate.

Growth with mixed substrates. During growth on mixtures of glutamate and glucose higher growth yields were obtained than with either substrate alone. It was of interest to determine whether the organisms exhibited a preference for one or other of the substrates under these conditions. The growth curves for *Clostridium tetanomorphum* grown on mixtures of glucose and glutamate occasionally exhibited a diauxic phase; more often a short exponential phase was followed by a phase of slower growth. Measurements of the concentrations of substrates in the medium at different times indicated that glutamate was metabolized first and that only when the glutamate had disappeared was there any significant decrease in the glucose concentration (Fig. 3). The same results were obtained whether the inoculum was derived from organisms grown with glucose or from glutamate.

Increasing the glutamate concentration relative to glucose had the effect of delaying further the onset of glucose utilization (Fig. 4). However, it appeared that glutamate was not solely responsible for this effect, because glucose was still fermented at a later stage in growth even though considerable concentrations of glutamate remained in the medium. Increasing the concentration of yeast extract had the same effect on the onset of glucose metabolism as did increased glutamate (Fig. 4). Thus it was realized that the yeast extract was serving as an energy source and direct measurement of the

utilization of glucose by organisms growing with yeast extract + glucose (but without glutamate) confirmed this: glucose was not used appreciably until about a quarter of the growth had occurred. No effect on the utilization of glutamate of doubling the yeast extract concentration was detected.

So, contrary to what might have been expected from the growth yield results, glucose was not the principal energy source during the initial period of growth, but rather it appeared to be fulfilling the role of an essential metabolite. Presumably, the amino acids in the yeast extract served as preferential energy sources. This implied

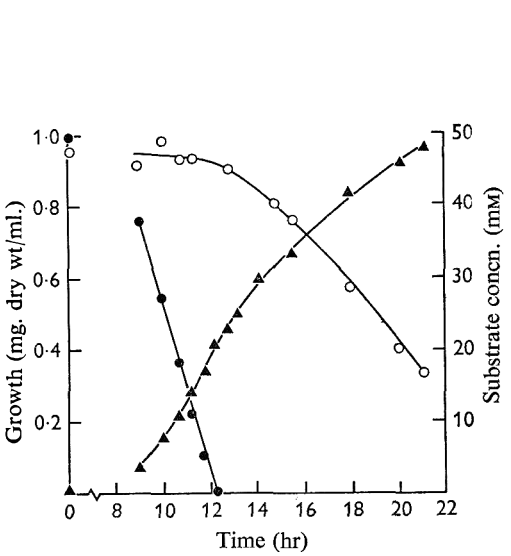

Fig. 3

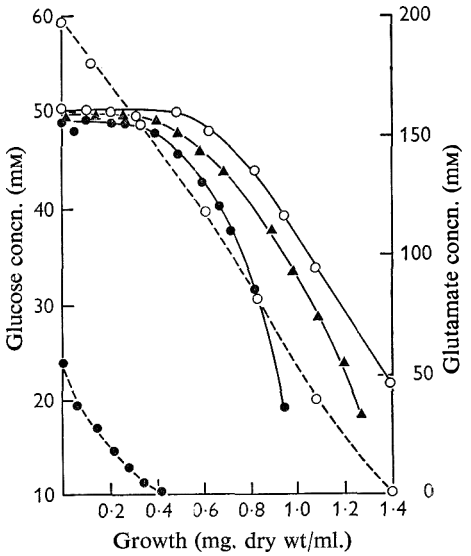

Fig. 4

Fig. 3. Growth of *Clostridium tetanomorphum* on a mixture of glutamate and glucose. The medium contained yeast extract (1·5 %) and the inoculum was 0·1 % (v/v) of a culture of organisms grown with glutamate. Concentrations of substrates remaining were: ●, glutamate; ○, glucose. ▲, Growth.

Fig. 4. The effect of glutamate and yeast extract concentrations on the utilization of glucose during growth of *Clostridium tetanomorphum*. Substrate concn.: ——, glucose; – – –, glutamate. ●, Glutamate initial concn., 0·05 M, yeast extract, 1·5 %; ○, glutamate initially 0·2 M, yeast estract, 1·5 %; ▲, glutamate initially 0·05 M, yeast extract 3·0 %. The initial concn. of glucose was 0·05 M in all cases and the inoculum was 0·1 % (v/v) of a glutamate grown culture.

that the mean generation time for growth with glucose, calculated during the early log phase, was not truly representative of the value for growth with glucose as the energy source. During growth with glutamate or pyruvate, the substrate was used from the medium at the rate expected from the growth yield results. Furthermore, with a mixture of glutamate and pyruvate, there was no increase in growth yield and both substrates were used simultaneously.

Induction of glucose and glutamate fermentations

A possible explanation for delayed glucose fermentation and preferential metabolism of yeast extract components is that the enzymes of the glucose pathway are inducible and that their formation is repressed by a constituent of the yeast extract or

by a product of its metabolism; derepression would then accompany depletion of the constituents from the medium. This would then be an example of catabolite repression (Magasanik, 1961). Alternatively, one or more steps in the path of glucose utilization might be inhibited by constituents or catabolites of the yeast extract; depletion of such compounds during the early phase of growth would annul the inhibition and permit glucose utilization.

To investigate these possibilities washed suspensions of organisms grown under different conditions were tested for ability to ferment glucose, glutamate or pyruvate. The rate of hydrogen evolution with these substrates under anaerobic conditions (Q_{H_2}), was taken as an index of their fermentative capacity. Suspensions of glucose-grown organisms produced hydrogen with glucose or pyruvate but not with glutamate; conversely, glutamate-grown organisms produced hydrogen with glutamate or pyruvate but not with glucose (Table 1). Pyruvate-grown organisms produced significant quantities of hydrogen only when incubated with pyruvate. The specific activity of a key enzyme of the Embden–Meyerhof pathway, phosphofructokinase, in extracts of organisms grown with glucose was four times greater than for organisms grown with glutamate, and the specific activity of β-methylaspartase, the second enzyme in the glutamate pathway, was about 12 times greater in extracts of glutamate-grown organisms (Table 1). Thus it appears that both the glucose and glutamate fermentations are inducible in *Clostridium tetanomorphum*.

Table 1. *Properties of organisms grown with different substrates*

Organisms were grown in medium containing substrate (0·1 M) and yeast extract (1·5 %). Q_{H_2} and enzyme specific activities were measured as described in the Methods section.

	Growth substrate		
	Glutamate	Glucose	Pyruvate
Q_{H_2}-glutamate	44	0	0
Q_{H_2}-glucose	0	15	0
Q_{H_2}-pyruvate	24	20	45
Phosphofructokinase	0·036	0·150	—
β-Methylaspartase	3·60	0·29	—

To determine whether the delay in glucose utilization was due to repression of the enzymes for glycolysis, the Q_{H_2} for glucose was measured for organisms withdrawn from cultures which had glucose present from the time of inoculation (Fig. 5). Glutamate-grown organisms were used as inoculum and following an early period of induction, during which less than 10 % of growth occurred, the Q_{H_2} for glucose remained more or less constant and there was no significant increase during the period in which glucose was being fermented at an increased rate. However, when glucose was added to a culture which had just exhausted a growth-limiting amount of glutamate, the Q_{H_2} for glucose increased from 0 to 13 (Fig. 6) and values of 7 and 8 were reached before the glucose concentration started to fall. Here again, the growth began before glucose was utilized. Concomitant with the induction of ability to ferment glucose there was an increase in the specific activity of phosphofructokinase (Fig. 6). These observations confirm that the glucose fermentation was inducible and, in addition, they indicate that control of glucose fermentation was achieved by a mechanism

other than enzyme repression, because even when the fermentation enzymes were present glucose was not utilized from the medium.

In similar experiments with organisms growing on glutamate+glucose from the time of inoculation, the Q_{H_2} for glutamate and the specific activity of β-methylaspartase were almost halved between the time of glutamate disappearance and the termination of growth. Such changes were consistent with a cessation of further synthesis of enzymes of the glutamate pathway during the period in which the growth yield doubled. Furthermore, when glutamate was added to a culture of *Clostridium tetanomorphum* growing with glucose, the rate of glutamate utilization increased rapidly

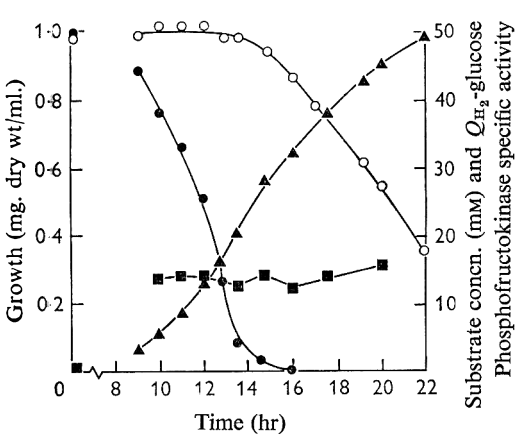

Fig. 5 Fig. 6

Fig. 5. The capacity to ferment glucose of organisms harvested at various phases of growth from a medium containing yeast extract (1·5 %) and a mixture of glutamate and glucose starting with an inoculum (0·1 %, v/v) grown with glutamate. ▲, Growth; ●, glutamate concn.; ○, glucose concn.; ■, Q_{H_2} for glucose.

Fig. 6. Induction of the ability to ferment glucose (Q_{H_2}-glucose), and of phosphofructokinase after the addition of glucose (28 mM at 13 hr indicated by the arrow) to organisms grown with glutamate (60 mM). ▲, Growth; ●, glutamate concn.; ○, glucose concn.; △, specific activity of phosphofructokinase; ■, Q_{H_2} for glucose.

and there was a marked increase in growth rate. Simultaneously, there was a progressive increase in the Q_{H_2} value for glutamate of samples removed from the culture after increasing periods of time.

Cobalamin content of Clostridium tetanomorphum. In view of the inducible nature of the glutamate fermentation it was of interest to determine whether the cobalamin content of the organisms was regulated in a co-ordinate fashion, because of the participation of cobalamin in the metabolism of glutamate. No marked differences in cobalamin content were observed for organisms grown with different substrates (Table 2). Even when the glutamate content of the glucose medium was decreased from the usual 5 mM to about 0·5 mM by previous treatment of yeast extract with glutamate decarboxylase, there was only a slight decrease in the cobalamin content. The values obtained with *Euglena gracilis* assay were about 20 times higher than those with the *Escherichia coli* 113/3 assay. This discrepancy was presumably because the organism synthesized predominantly the coenzyme form of pseudo-vitamin B_{12} and although

pseudo-vitamin B_{12} and cyanocobalamin (vitamin B_{12}) are equally active for *E. gracilis* previous reports indicate that pseudo-vitamin B_{12} is only one-tenth as active for *Escherichia coli* 113/3 (Smith, 1965).

Table 2. *Cobalamin content of Clostridium tetanomorphum harvested from different media*

Organisms were grown in media containing 1·5 % yeast extract and the substrate indicated (0·1 M). Assay of cobalamin was as described in the Methods section.

	Cobalamin content (μmg./mg. protein)	
Substrate	*Euglena gracilis*	*Escherichia coli* 113/3
Glutamate	256	12·1
Histidine	274	12·9
Pyruvate	326	14·8
Glucose	245	13·6
Glucose*	190	10·2
Maltose	234	8·4

* The yeast extract used for preparing the medium was treated with glutamate decarboxylase to remove glutamate.

DISCUSSION

Until recently very little was known about the control of metabolic processes in strict anaerobes, and it appeared that *Clostridium tetanomorphum* would provide a suitable subject for such a study. However, contrary to preliminary indications that single substrates would serve as principal energy source, it is now clear that this organism has considerable nutritional complexity. The apparent discrepancy between the observation that the final growth yield may be proportional to the initial glucose concentration, and the observation that the extent of glucose utilization at a given time during growth is not proportional to the amount of growth, makes it difficult to be certain at any time whether a substrate is functioning as a principal energy source. The importance of the yeast extract as an energy source cannot be overlooked. Furthermore, when growth has ceased for reasons other than lack of energy sources, it is possible that an uncoupled metabolism of substrate may occur.

Glutamate, pyruvate and glucose all promoted the growth of *Clostridium tetanomorphum* in the yeast extract medium. However, glutamate and pyruvate were metabolized without appreciable delay, whereas glucose utilization was deferred. The delay in glucose catabolism suggests that glucose promotes growth primarily by serving as a carbon source or growth factor and that the organism possesses a regulatory mechanism for glucose conservation. The role of glucose as energy source was of secondary importance during the early part of growth since components of the yeast extract were used preferentially for this purpose. In addition to glutamate and histidine (which permit growth), the energy-yielding components of the yeast extract undoubtedly include aspartate, serine, cysteine, methionine and tyrosine, which are known to be fermented by washed suspensions of this organism (Woods & Clifton, 1938). Rapid metabolism of these compounds via pyruvate would make a significant contribution to the amount of energy available during the initial stages of growth, when growth is not prevented by lack of a suitable carbon source. By contrast with glucose the early

metabolism of glutamate and pyruvate suggests that these are preferred energy sources which may promote growth by sparing the carbon sources or essential metabolites in yeast extract. The absence of growth with yeast extract alone could thus be interpreted as due to a deficiency of carbon sources, or to a lack of sufficient energy sources.

In yeast the glycolytic enzymes are induced by increasing the glucose concentration in the medium (Hommes, 1966) and, Lee & Ordal (1967) have shown that *Clostridium thermosaccharolyticum* has increased amounts of glycolytic enzymes but a lower rate of pyruvate fermentation when grown with glucose as compared, with pyruvate. In the present work with *C. tetanomorphum* both the glycolytic and the glutamate pathways were inducible and the capacity to ferment pyruvate was somewhat lower in organisms grown with glutamate or glucose as compared with the capacity in organisms grown with pyruvate. That pyruvate is fermented by glutamate or glucose-grown organisms is to be expected since 'phosphoroclastic' metabolism of pyruvate is thought to be common to glutamate, glucose and pyruvate fermentations.

No evidence has been obtained here for the existence of a repression mechanism for the glutamate pathway of *Clostridium tetanomorphum*. Glutamate metabolism appears to be unaffected by the presence of pyruvate, glucose or increased concentrations of yeast extract. Similarly, the results indicate that the regulation of glucose metabolism is not due to catabolite repression by glutamate or some component of the yeast extract, because the capacity to ferment glucose was induced even in their presence. Nevertheless, the onset of glucose metabolism was deferred in organisms possessing the capacity to ferment glucose, so it appears that the control mechanism resides in the inhibition of one or more enzymes of glycolysis by a metabolizable constituent of the medium or a product thereof.

Many mechanisms might be suggested to account for this feed-back or catabolite inhibition. It seems unlikely that pyruvate is directly responsible for the inhibition because tests have shown that there was no significant increase in the concentration of pyruvate during the period of deferred glucose metabolism. Nor could glutamate be solely responsible because glucose metabolism was initiated in the presence of excess glutamate. Some component of the yeast extract might have a direct inhibitory effect on one of the enzymes of glycolysis or on a glucose permease. However, a more likely explanation is that glycolysis in *Clostridium tetanomorphum* is regulated by a feed-back inhibition of one or more of the glycolytic enzymes by a catabolite of a yeast component. Such feed-back mechanisms have been found in other micro-organisms and in higher organisms. Possible examples are the inhibition of phosphofructokinase by ATP and citrate (Passonneau & Lowry, 1964; Lowry & Passonneau, 1964) and of pyruvatekinase and gluconokinase by acetylCoA (Weber, Lee & Stamm, 1967). Also in this context a delay in the removal of glucose from the medium by *Escherichia coli* has been thought to be due to a product of pyruvate metabolism (Morgan & Kornberg, 1967). The absence of any marked variation in the cobalamin content of *C. tetanomorphum* grown in different media suggests that synthesis of the vitamin is not co-ordinated with the fermentation of glutamate. A regulatory mechanism may involve an influence of glutamate on the conversion of cobamides to the coenzyme forms, or alternatively the vitamin may have as yet unknown functions in the metabolism, for example of other amino acids, and enzymes for its synthesis may therefore always be induced in the medium used here.

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A New Type of Micromanipulator and Microforge

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INTRODUCTION

The need for instruments which can be used for the manipulation of microscopically small objects has led to the invention of several micromanipulators. The more refined of these are research tools, the high cost of which matches their precision and mitigates against their general use, particularly by undergraduate students. With more attention being paid to genetics and the study of microcolony development in bacteria and unicellular algae, a greater interest has developed in the separation of single cells from microcolonies and in the selection of microcolonies themselves to prevent overgrowth by a more dominant population. This has increased the need for a simple form of micromanipulator and microforge. The author possesses the hydraulically operated de Fonbrun micromanipulator and microforge and also a Zeiss Jena micromanipulator. Both of these are excellent instruments but their design, apart from cost, makes difficult the handling of tools within the confines of the normal Petri dish. A new type of micromanipulator and microforge has therefore been designed which makes use of the optical systems normally available in the modern laboratory. Patent rights have been applied for in most leading countries.

THE MICROMANIPULATOR

This instrument was designed for use with the Reichert low-power ($\times$ 10) objective but can be used with suitable modification with any low-power objective with a working distance of about 7 mm. or greater. It can be used with a normal light-field objective but a phase-contrast objective is to be preferred. It consists of two parts. One, for convenience, is called the *lens collar* (Fig. 1) and the other the *magnetic tool-carrier* (Fig. 2). The assembled micromanipulator is shown in section in Fig. 3.

The lens collar

This consists simply of a non-ferrous metal (preferably brass) collar *A* with a centre hole *B* machined to fit closely but not too tightly to the outer casing of the low-power objective and secured to the objective casing with a non-metal screw *C*. A plastic liner (not shown) improves the fit. Attached to the side of the collar are two steel slides *D* machined parallel and perfectly flat on the under surface. These slides are spaced equally on either side of the centre axis of the collar on the side opposite the retaining screw *C*. The width of the slides is determined by the width of the small magnet *H* (Fig. 2) and the tool-carrying tube *J*. The inter-slide spacing *E* should be not less than 3 times the outer diameter of the tool-carrying tube *J*, to permit adequate

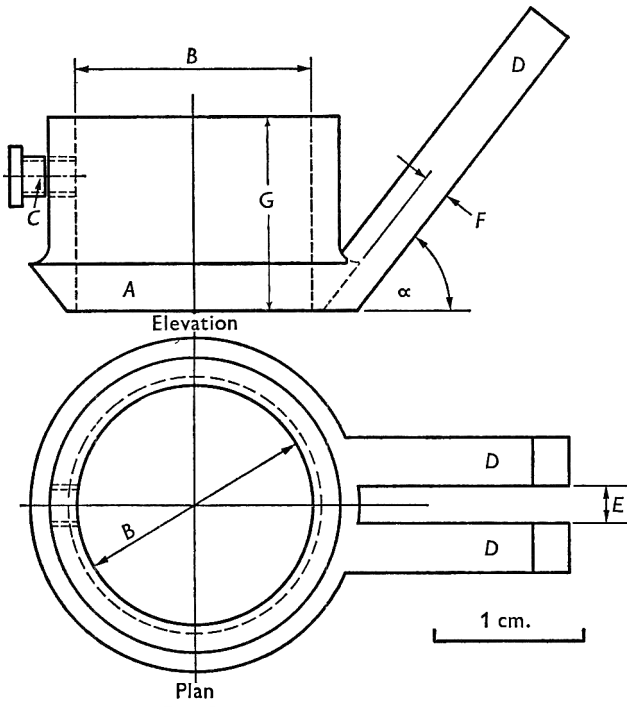

Fig. 1. Lens collar. For explanation see text.

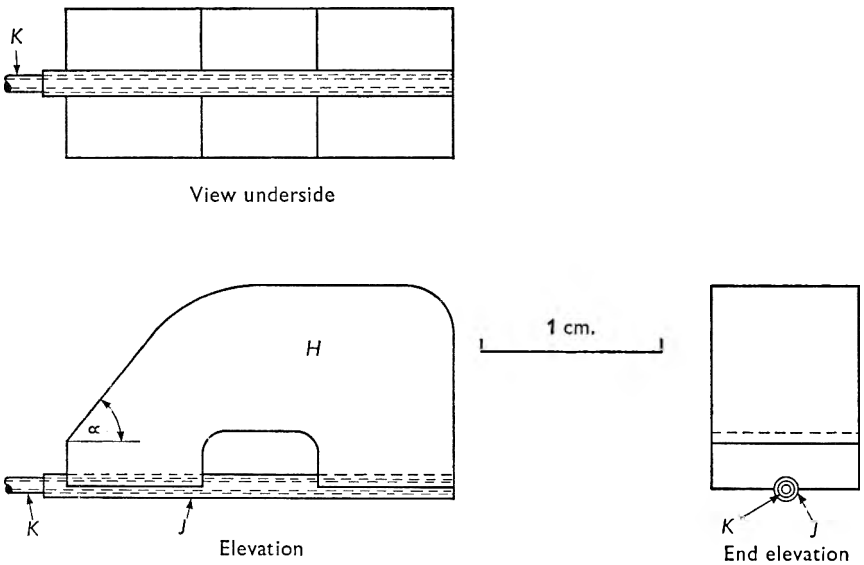

Fig. 2. Magnetic tool-carrier. For explanation see text.

lateral movement in positioning the tool. The distance between the outer edges should not be less than the width of the magnet and preferably equal to it.

The thickness of the steel slides and their mode of attachment must be such that the clearance between the under surface of the slides and the metal collar (dimension *F*) is at least equal to the diameter of the tool-carrying tube *J*. This permits the magnet to slide freely along the steel slides without the tool-carrying tube *J* fouling the lower edge of the collar. The angle of attachment of the steel slides must be such that when the magnet is in position the *projected* uppermost edge of the attached tool-carrying tube *J* does not contact any of the metal parts of the objective when the projected centre axis of the tool-carrying tube *J* is made to pass through the focal point of the front objective lens. For ideal operation the clearance should be at least 1·5 mm.

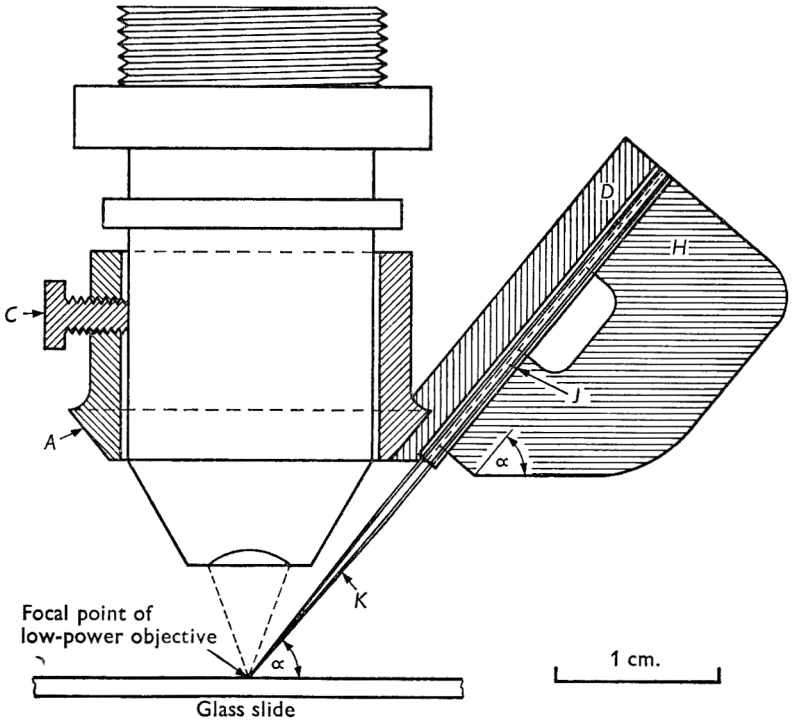

Fig. 3. Schematic drawing of micromanipulator in position. For explanation see text.

The height *G* of the metal collar *A* should be such as to provide a secure attachment of the lens casing and to allow vertical movement of the (projected) centre axis of the tool-holder at least 1 mm. above and below the focal point of the objective. The thickness of the collar should be adequate to provide a secure attachment for the screw *C*. If not, a metal extension should be provided for the attachment of the screw.

The magnet tool-carrier

The magnet *H* may be of any dimensions but preferably should not exceed 25 mm. in length or 10 mm. in width or 12·5 mm. in depth. The depth of the magnet should be sufficient to ensure that it can be easily gripped for manipulation between the thumb and index finger. An Eclipse Minor Magnet (catalogue no. 801) manufactured

by James Neill and Co. (Sheffield) Ltd., England, has proved very satisfactory. When a magnet of this type is used one end should be truncated at the angle α to ensure that its lowest point, when in the operative position, does not project near or below the focal point of the objective.

The tool-carrying tube *J* is a cylindrical tube of any diameter but preferably not exceeding 1 mm. outer diameter and constructed of stainless steel. A section of a 19-gauge hypodermic needle has been used by the author, and it should exceed the length of the magnet by about 1 mm. It is recessed into the face of the magnet along the centre line and cemented to it with some material which will permit replacement of the tubing when required. The depth of the recess is not important, but when glass tools are to be used and are to be fabricated on the microforge the depth of the recess should be such that the centre axis of the tool-carrying tube *J*, *when cemented into position*, lies in the same plane as the face of the magnet.

The tool *K* may be of glass or other material. For some purposes platinum wire, of the maximum gauge which will fit into the tool-carrying tube *J*, may be used and secured into the tool-holder with some malleable wax. The length of the extruded portion of the wire should be approximately equal to the distance between the lower end of the steel slides and the focal point of the objective when the tool is in the operating position. The tip of the platinum wire should be ground to a point on a fine honing-stone.

When glass tools are to be used good-quality soda-glass (preferably, since Pyrex is too brittle) tubing or rod should be drawn out to a diameter slightly smaller than the internal diameter of the tool-holder. A piece of this drawn tubing or rod is secured into the tool-holder with paraffin wax and severed from the end of the tool-carrying tube *J* at a distance approximately equal to the distance between the lower end of the steel slides and the focal point of the lens when the tool is in its working position. The glass can be worked into any desired form with the microforge (Fig. 4).

Operation

The micromanipulator, without the magnetic tool-holder $H+J$, is secured to the low-power objective in the lowest position compatible with stability. The magnetic tool-holder *HJ*, complete with tool *K*, is then attached to the steel slides and pushed down along the slides *D* until the tip of the tool passes slightly beyond the centre of the lens. While viewing through the microscope, collar *A* is slowly raised on the lens casing until the image of *any* part of the tool is brought to focus in the *centre* of the field. (It may be necessary to move the magnet slightly from side to side to align the tool—hence the gap between the steel slides.) The magnet is then drawn back up the steel slides until the tip of the tool *K* is in the centre, when a final adjustment of the collar may be necessary. The magnet is then withdrawn further up the slide while a search is made for the specimen to be manipulated. Since the primary purpose in the design of the manipulator was the selection of single organisms or groups of organisms from microcolonies of bacteria, the colony is brought into focus in the centre of the field. The barrel of the microscope is then raised and the magnet pushed down till the tip of the tool returns to focus in the centre of the field. The microscope is then refocused on the colony. When this is done the tip of the tool makes contact with the centre of the colony. By re-positioning the specimen just before the colony comes into precise focus it is possible to manipulate from any desired area.

The precise mode of manipulation depends on the design of the tool, which may be of a great variety of shapes. Three simple tools are the sharp probe, the knob, and the microloop. Of these the knob and the loop are the most useful for single-organism isolations, the probe for marking the position of an organism on the agar surface.

Single-organism isolation by probe or loop, from a butyrous type colony, can be achieved simply by lowering the tool onto the edge of the colony, lifting it free from the agar, moving the Petri dish with the mechanical stage (modified to grip the perimeter of the dish), followed by lowering the tool again onto the uninoculated agar surface. A succession of such motions will rapidly thin out the population, finally resulting in the deposition of one organism or a small group of organisms.

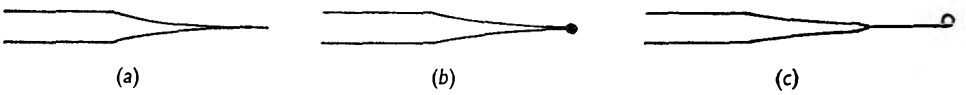

Fig. 4. Three simple tools: (*a*) sharp probe, (*b*) knob, (*c*) microloop.

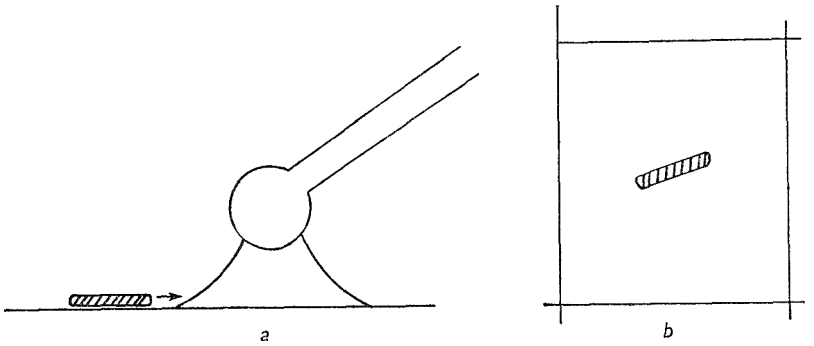

Fig. 5. *a* Microtool pressing on agar. *b* Position of organism marked by scoring surface of agar with probe.

At this stage the microtool should be replaced by a sterile tool, preferably a knob. When this is pressed down on the agar to one side of an organism, liquid will be expressed from the agar and the organism can be encouraged to float into it (Fig. 5 *a*). Once this has occurred careful lateral movement of the Petri dish will permit the single organism to be floated across the agar, well clear of the original population. The position of this organism can be marked by scoring the surface of the agar with the probe (Fig. 5 *b*). After incubation the developing colony can be transplanted. Alternatively, the small block of agar supporting the organism can be excised under a stereo-microscope by using a sterile scalpel.

Attempts to lift *single organisms* in a loop for transfer to a new Petri dish are rarely successful, because of the rapid drying-up of the fluid. Transfer of *masses of organisms* in this manner can usually be effected.

THE MICROFORGE

The manufacture of precise glass tools for use with the micromanipulator necessitates the use of some tool-fabricating device, of which several are marketed. The microforge described here makes use of the principle used in the de Fonbrun microforge and only one portion of the assembly here described (iii) is claimed to be original. The assembly can be used on any microscope fitted with a mechanical stage. It consists essentially of three parts.

A regulated power supply. For this purpose the following electrical circuit shown in Fig. 6 is used.

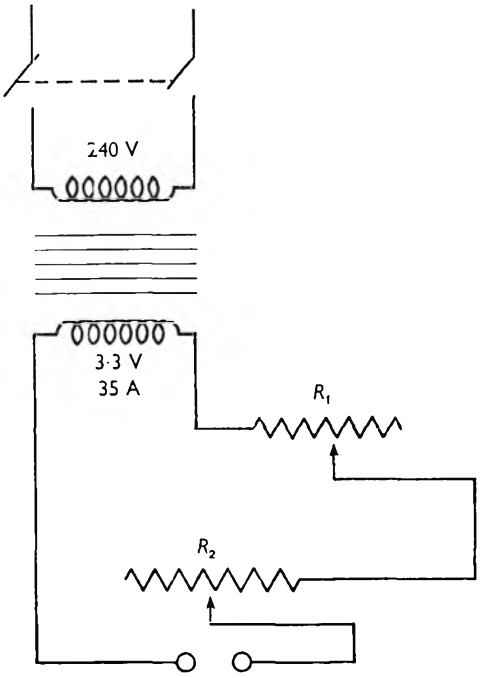

Fig. 6. Circuit for regulating power supply.

The transformer used is one manufactured by National Transformers Pty. Ltd., Australia (primary 240 V 50 cycles; secondary 3·3 V; 30 A intermittent rating). The variable (7·07 A) resistance R_1 is a 1Ω Type L50 CF manufactured by the British Electric Resistance Co. Ltd., Enfield, Middlesex, England. Variable resistance R_2 is a 2 Ω Type L 100 (7·07 A). The sole purpose of resistance R_1 is to limit the maximum voltage across the element L (Fig. 7; a 15 mm. length of platinum/iridium wire) to a value which will not fuse the wire. Control of heating within this range is obtained with the variable resistance R_2.

The platinum/iridium heating stage (Fig. 7). The design of the holders M for the platinum/iridium wire element L is essentially the same as that of the de Fonbrun microforge. They are mounted in an insulated block N on a metal slide O and operated on an ordinary microscope by means of a mechanical stage. The height of the element

L above the slide *O* must be greater than the distance between the centre of the hole *X* and the base of the vertical support *V* in Fig. 8 and 9.

Microforge tool-carrier. The assembled microforge is shown in sectional diagram in Fig. 8 and in exploded form in Fig. 9. The tool-carrier consists essentially of the three parts described below.

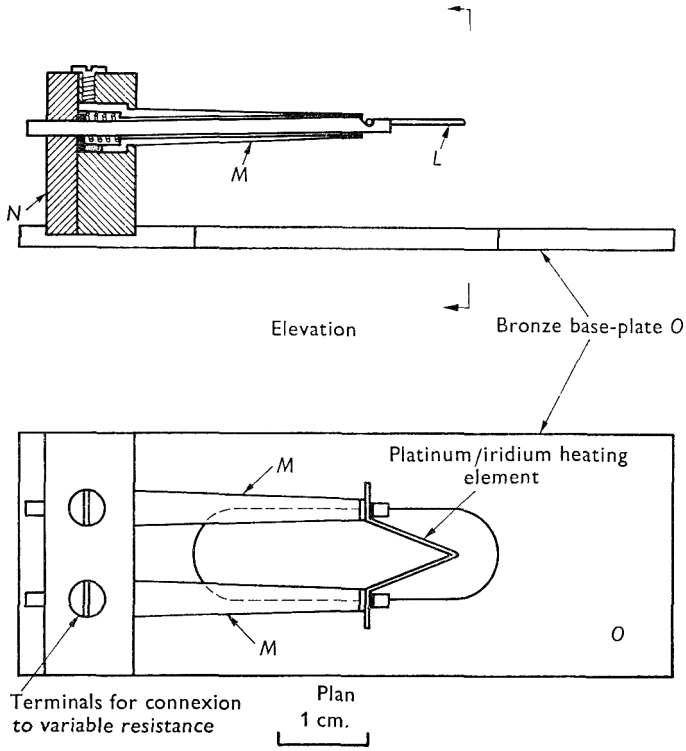

Fig. 7. Platinum/iridium heating stage. For explanation see text.

(i) A collar *P* whose internal diameter is determined by the external diameter of the casing of the ×2·5 objective lens *Q* to which it is secured by a plastic screw *S*. The thickness of the wall must be such as to provide a secure thread base for the plastic screw. At the base of the collar is a flange *T* upon which the ring *U* rotates. Vertical adjustment of the position of the collar *P* on the casing of the lens permits the glass tool *K* to be brought to focus at the focal point of the objective lens when the ring *U* is seated on the flange *T*.

(ii) A ring *U* to which is attached the vertical support *V*. The internal diameter of the ring should be such that it fits closely but not too tightly to the collar *P*. The thickness of the ring should be such as to permit the fixing of the vertical support *V* and to separate it from the flange *T* at the base of the collar *P* by about 1 mm. The height of the ring *U* should be sufficient to ensure a rock-free fit on the collar and adequate space for the insertion of the plastic screws W1 and W2 in the ring. The plastic screws in the ring should (for convenience only) be located opposite to and at right angles to the vertical support.

A hole X, 5 mm. in diameter, is drilled through the brass supporting plate V which is positioned on U so that the centre of the hole lies on a line in the same plane as the centre of the ring U. The plate V is milled on its outer face to a depth sufficient to receive a polytetrafluoroethylene (PTFE) washer and the circular plate Y_1 at the end of the steel carriage Y. The steel carriage is secured to the supporting plate V by a cover plate Z and four screws, the cover plate itself being drilled and recessed on its inner face to receive a second PTFE washer. When secured in position the flat surfaces of the steel carriage Y are at right angles to the central axis of the supporting ring U and the supporting plate V and are in the same plane as the centre of the hole X.

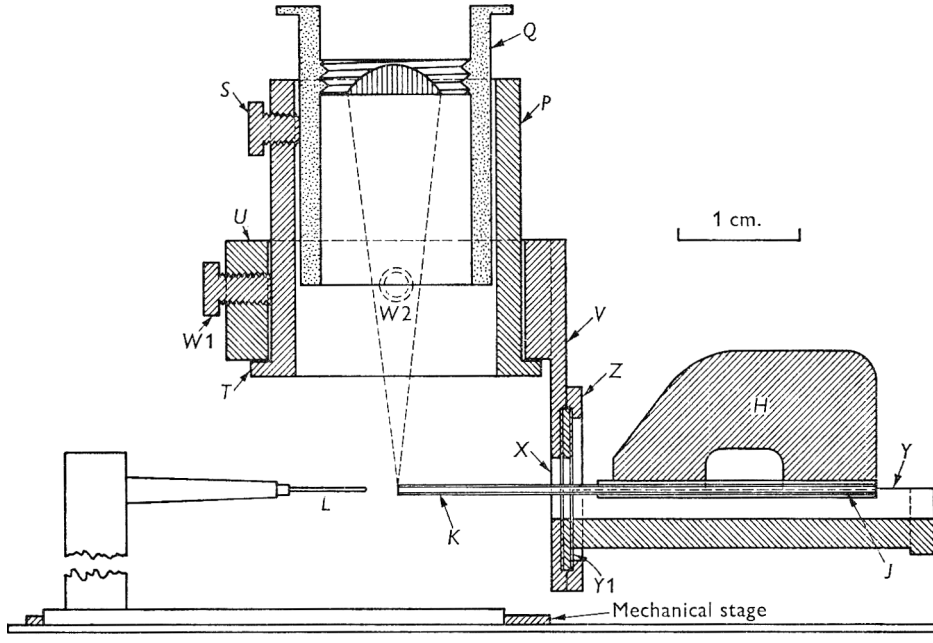

Fig. 8. Schematic drawing of microforge in working position.

(iii) A steel carriage Y, machined from a single piece of cylindrical light steel as illustrated, the carriage being a fraction wider than the magnet H used in the manufacture of the magnetic tool-carrier and its length about 12·5 mm. longer than the length of the magnet.

The over-all dimension of the completed microforge tool-carrier must be such that, when attached to the microscope lens, it can be raised or lowered to a position where the horizontal plane through the centre of the hole X in the vertical support V will pass through the focal point of the objective. The diameter of the ring Y_1 at the end of the steel support Y should be as large as is compatible with the over-all dimensions of the tool in order to achieve maximum stability. When assembled the steel carriage should rotate freely in the support but without any slackness.

The lens used to support the microforge is a $\times 2{\cdot}5$ lens with a specially attached collar to support the microforge. The author has used a $\times 10$ objective from which the terminal lens had been removed.

Operation of the microforge. The steel carriage *Y* is rotated until its flat surface is parallel to the surface of the stage. The magnetic tool-carrier *H*+*J*, fitted with a glass rod *K*, is placed on the free end of the steel carriage *Y* and slipped forwards so that the glass rod passes through the centre of hole *X* and the end of the magnet comes

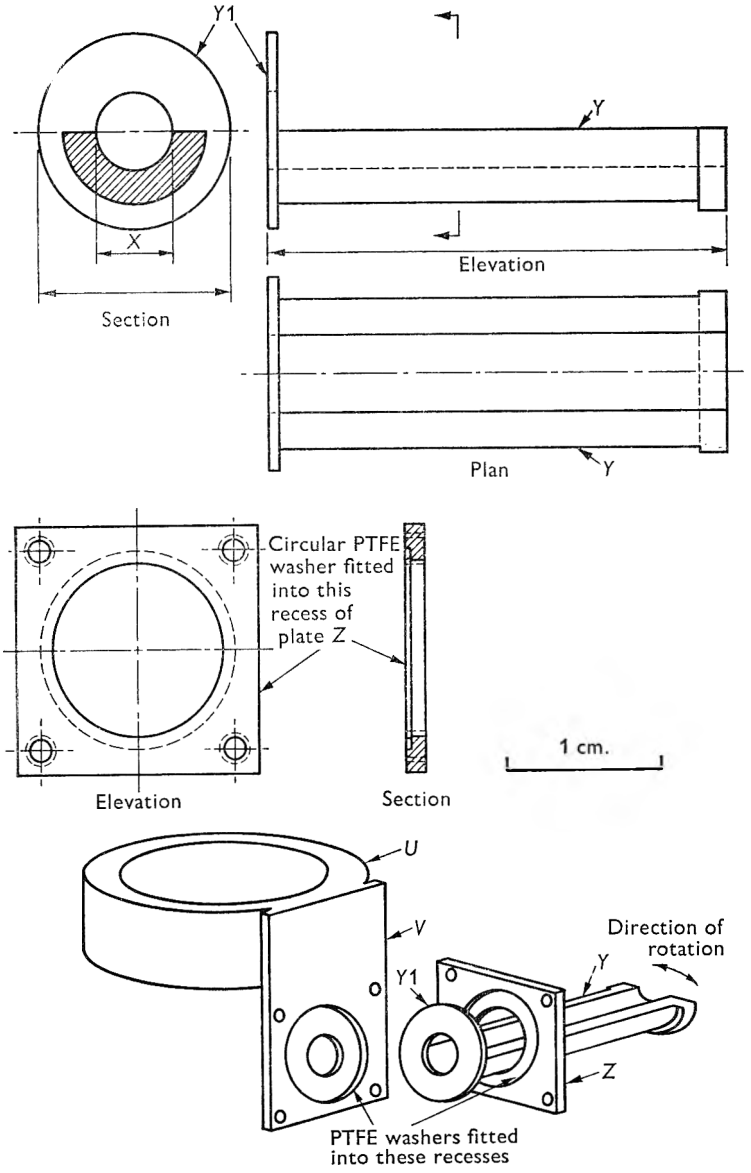

Fig. 9. Microforge.

close to the cover plate *Z*. The height of the collar *P* is adjusted on the lens casing until the glass rod is brought into focus. The magnet is then retracted until the tip of the glass rod is in the centre of the field; when necessary the focus is readjusted. Provided the glass rod is centrally located it should be possible to rotate the steel carriage *Y*

through 90° either way without the rod going out of focus. Normally some minor adjustment to the magnet will be necessary.

The magnet is then returned to the vertical position and the carriage Y aligned with its long axis parallel to the front end of the microscope stage. Whilst inspecting the instrument from the front of the microscopic stage, the heating stage (Fig. 7) is moved with the mechanical stage until the tip of the heating element L is directly opposite and close to the tip of the glass rod K. Then, whilst viewing through the microscope, the height of the barrel of the microscope is adjusted until the platinum/iridium heating element L is brought to focus in a position opposite to the end of the glass rod. By the application of current to the platinum/iridium wire the glass is melted and drawn out to a fine point by withdrawing the heated element with the mechanical stage.

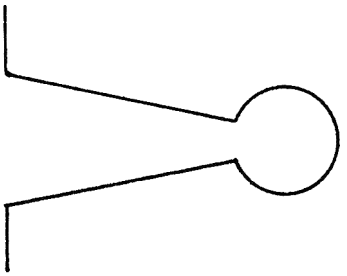

Fig. 10. Shape of platinum wire.

Glass microloops can be manufactured (full details can be obtained from the author by those using the instrument) by the same procedure as that described by de Fonbrun, the temperature of the platinum/iridium wire being controlled with resistance R_2. With the apparent closure of the loop the flatness can be checked by rotating the steel carriage Y through an angle of 90°. Any adjustment to the plane of the loop can be made in this position. To bend the loop at the angle required for use on the micromanipulator the plastic screws $W1$ and $W2$ securing the ring U to the collar P are relaxed and the ring rotated so that the steel carriage as a whole passes through the required angle towards the front of the stage. The screw $W2$ is then tightened and the shaft of the loop bent near the base of the loop by gentle pressure with the warm platinum/iridium wire.

To prepare capillaries a procedure similar to that used by de Fonbrun can be used. The microscope is inclined backwards until the barrel is horizontal. It is then anchored on a support which will permit comfortable inspection through the ocular whilst in a sitting position. A glass capillary is fitted into the tool holder and the free end bent into a hook. With the ring U rotated so that the glass rod is in a vertical position, weights can be applied to the hooked end and the glass rod heated from the side in the manner described by de Fonbrun. A refinement of the heating element, which aids in the manufacture of capillaries, is one which permits the holders of the platinum/iridium wire to rotate through 90°. The platinum wire itself is bent as in Fig. 10. With the loop horizontal to the stage it is positioned so that the centre of the ring is precisely in the centre of the field and in focus with the lens. Allowance must be made for the

lateral expansion of the heated wire. The loop is then rotated through 90° and the glass rod pushed down so that it becomes positioned through the centre of the ring. Weights are applied and the element heated. Whilst capillaries can be manufactured quite easily their use in the magnetic tool-carrier presents difficulties which have still to be resolved. The author has found little use for capillaries. Most single-organism isolations can be achieved more quickly by a probe, a probe with a small knob at the end, or a loop.

Once a tool has been prepared the magnet is removed carefully from the forge and applied directly to the slides of the manipulator. Preparation of a new probe, once a probe has been used, simply involves melting the used probe and drawing a new one. The time required is less than that needed to heat and cool a platinum or nichrome wire. The temperature reached ensures sterility. Microloops can be prepared in about two minutes.

DISCUSSION

The whole apparatus has the advantage of being simple in design, small in size, and adaptable to use on any microscope. The design of the microforge gives three-dimensional control over tool manufacture and yields a finished glass tool mounted in the magnetic carriage in which it is subsequently used on the manipulator, thus permitting rapid operation. Used with a low-power phase-contrast lens, single-organism isolations can quite easily be effected with *Bacillus* sp. and organisms of similar or greater dimensions. Use of the manipulator with a high-power ($\times 40$) lens presents problems, not the least of which is condensation of moisture from the agar on the lens surface, for which there is no cure. However, a manipulator of slightly altered design, in which the slides *D* are fixed a little above the horizontal (angle α in Fig. 1 is almost 0) and the magnet is mounted on top with an undercarriage which ensures that the centre of the tube *J* is in the same plane as the under surface of the slides *D*, can be used on moderately dry surfaces. Other modifications which would permit manipulation under a cover glass are being considered.

The problem of dust falling on exposed Petri dishes during manipulation has been countered in this laboratory by working with the microscope positioned under a polythene hood with the eyepieces protruding through the wall. The working area is sterilized by means of an ultraviolet light. Clarified agar is used as a matter of routine in any micromanipulative work.

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Production of Axenic Cultures of Soil-borne and Endophytic Blue-green Algae

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SUMMARY

A relatively rapid and reliable method is described for producing axenic cultures of soil-borne blue-green algae, of the blue-green algal endophytes of certain cycads, and the blue-green endophyte of the angiosperm *Gunnera chilensis* Endl. It was based on the micro-manipulation of hormogonia and akinetes, from which axenic cultures were propagated. Micromanipulation was done during growth in impure culture on an agar medium. The technique was used to obtain axenic cultures of fifteen soil isolates, the endophytes of the cycads *Macrozamia lucida* (Linnaeus) Johnson, *Encephalartos altensteinii* Lehm., and *Bowenia serrulata* Bull, and the endophyte of *Gunnera chilensis* Endl. The soil isolates have been tentatively identified as species of the genera *Nostoc*, *Anabaena*, *Calothrix*, *Scytonema*, *Wollea*, *Microchaete*, and *Oscillatoria*.

INTRODUCTION

The blue-green algae have been found exceptionally difficult to obtain in axenic (pure) culture, due largely to their characteristic mucilaginous sheaths which harbour contaminating bacteria (Fritsch, 1945; Pringsheim, 1946). Thus protected, the bacteria are virtually impossible to eliminate by standard microbiological plating and dilution techniques. A variety of approaches has been tried for producing axenic cultures, but in all instances they have involved trial and error techniques, very dependent upon the chance removal of microbial contaminants.

Ultra violet (u.v.) irradiation, to which blue-green algae have been found relatively resistant, has been the most widely used method for obtaining axenic cultures (Allison & Morris, 1930; Allison, Hoover & Morris, 1937; Gerloff, Fitzgerald & Skoog, 1950; Fogg, 1951; Holm-Hansen, Gerloff & Skoog, 1954; Venkataraman, Dutta & Natarajan, 1959; Venkataraman, 1961; 1962). Kraus (1966) produced axenic cultures by the use of gamma radiation, to which blue-green algae were also found to be relatively resistant. Other techniques which have been used include: (*a*) repeated sub-culture on a silica gel + mineral salts medium (De, 1939; Fogg, 1942, 1944; Henriksson, 1951; Watanabe, 1951); (*b*) methods based on phototactic and migration responses of blue-green algae (Schramm, 1914; Allen, 1952; Bunt, 1961); (*c*) use of antibiotics, successfully for one species by Pintner & Provasoli (1958), but found to be of no value by Tchan & Gould (1961); (*d*) use of disinfectants such as dilute chlorine water (Fogg, 1942, one species) and dilute phenol solution (McDaniel, Middlebrook & Bowman, 1962, one species); (*e*) treatment of one species at a pressure of 12,000 lb./sq. inch, which destroyed bacteria and fungi, but had relatively little

destructive effect on the alga (Middlebrook & Bowman, 1964); (*f*) relation of the problem to a binomial distribution function, and resultant use of a very laborious method based on dilution and replication (Tischer, 1965; two species). That chance plays a major role in these techniques is illustrated by the fact that Stewart (1962), who used a combination of radiation, antibiotic and plating methods, obtained an axenic culture of Oscillatoria only after 17 months. Further, the use of u.v. or gamma radiation may be criticized on the grounds of their possible mutagenic effects. Kumar (1963) and Singh & Singh (1964) produced strains of blue-green algae resistant to u.v. radiation, the use of which could conceivably lead to the selection of an axenic mutant of the original organism to be purified.

For these reasons, a method was sought which would enable the direct selection of uncontaminated algal material, from which axenic cultures could be propagated. The obvious approach was the use of a micromanipulator. This was tried by Gerloff *et al.* (1950), who were able to isolate only one species. Watanabe & Kiyohara (1963), who used a micromanipulator and antibiotic-containing media, were able to purify only 7 of 14 isolates from various lichens, liverworts and cycads. However, in neither instance were details of the micromanipulative procedures given.

The technique to be described was developed as a result of the necessity to obtain axenic cultures for the study of nitrogen fixation by blue-green algae. The micromanipulator used throughout the work was developed by Skerman (1968).

METHODS

In outline, the isolation technique involved growth of the impure algae on a mineral salts–agar medium, from which uncontaminated material was selected by micromanipulation, then transferred to a silica gel medium, in which the axenic cultures developed.

The medium used was like that described by Allen & Arnon (1955), except that the elements of their medium not yet shown to be required for growth were omitted. The formula was: macronutrients (g./l. glass distilled water) $MgSO_4.7H_2O$, 0·25; K_2HPO_4, 0·2; $CaCl_2$, 0·056; KNO_3, 0·5; micronutrients (mg./l.) (the metal equivalents, in mg./l., are given in parentheses) ferric monosodium salt of EDTA, 33·0 (Fe = 5·0); $MnCl_2.4H_2O$, 1·81 (0·5); $Na_2MoO_4.2H_2O$, 1·469 (0·1); $ZnSO_4.7H_2O$, 0·222 (0·05); $CuSO_4.5H_2O$, 0·079 (0·02); H_3BO_3, 2·86 (0·5); $Co(NO_3)_2.6H_2O$, 0·049 (0·01). Analytical grade chemicals were used. For solid media in early experiments, Difco Bacto agar was added at 1·5% (w/v). Oxoid Ion agar No. 2 was later substituted at 1·3% (w/v). The medium was adjusted to pH 7·5, and it was sterilized at 121° for 15 min. The silica gel medium was prepared in 20 ml. quantities in screw-capped bottles or in Petri dishes, by mixing equal volumes of the above mineral salts solution prepared at double concentration, and 3·0% (w/v), silicic acid, prepared according to the method of Pramer (1957). Tap-water agar was prepared by dissolving 15 g. of Difco Bacto agar in 1 l. tap water, and autoclaving at 121° for 15 min.

Isolation procedures

(i) *Primary isolation from soil.* Soil was collected in sterile screw-capped bottles from the surface half-inch of a Black Earth under fallow (Darling Downs, Queensland), by aseptic technique. In an attempt to select nitrogen-fixing species, 1 g. soil was

added to 10 ml. liquid medium, lacking combined nitrogen, in 50 ml. Erlenmeyer flasks, which were incubated under continuous fluorescent light of 800 lux intensity at the level of the cultures, at 25–30°. After 3 to 4 weeks some flasks developed dense growths of filamentous blue-green algae, which were then subjected to purification procedures.

(ii) *Primary isolation of endophytes.* Pieces of alga-containing cycad coralloid roots were surface-sterilized by immersion in 70% (v/v) ethanol in water for 1 min., then in 10% (w/v) sodium hypochlorite solution for 5 min., followed by repeated washing with sterile distilled water. Sections about 1 mm. thick were cut with a sterile scalpel and placed on the silica gel medium in Petri dishes.

Pieces of stem tissue containing the endophyte of *Gunnera chilensis* were surface-sterilized as above. The algal mass was then dissected out as aseptically as possible, and plated in small fragments onto silica gel medium. Plates were incubated under continuous fluorescent light, as with the soil flasks. Colonies of blue-green algae developed as concentric rings around the coralloid root sections and the Gunnera tissue in 3 to 5 weeks (Pl. 1, fig. 1–3).

After primary isolations, the procedures for purification of both the soil and endophytic species were the same. The soil cultures in particular were very crude, fungi and amoebae being the principal contaminants, with large numbers of bacteria. Fungi and amoebae were absent from many of the endophytic isolations, presumably as a result of the initial surface sterilization of the host material. When inoculated onto the agar medium, the crude soil isolates were frequently overgrown by fungi and/or amoebae. For this reason cultures free from the latter organisms were first obtained, before attempting to obtain bacteria-free cultures, as follows.

Small pieces of crude algal growth were inoculated onto agar plates, and incubated under continuous illumination for 1 to 2 days, by which time fungal and amoeboid contamination had developed. By using a microprobe, and × 10 phase-contrast objective, as large an area of peripheral algal growth as possible was marked out, disregarding bacterial contamination, but taking care to exclude fungi and amoebae. The agar blocks were cut out with a fine sterile scalpel under a stereoscopic microscope, and transferred to glass culture vessels (3 ml. capacity). These vessels were made by cutting 22 mm.-diameter flat bottomed tubes to 11 mm. in height, so that they just fitted between the base and lid of a plastic Petri dish. Three such cups were set in each dish in 15 ml. of tap-water agar. A few drops of liquid culture medium were added to each cup, so that the algal growth on the surfaces of the agar blocks was just submerged. A pellicle of algal growth usually developed on the liquid surface after 10 to 14 days under continuous illumination of 800 lux. This growth was then used for producing bacteria-free cultures, the details of which are as follows.

Petri dishes of agar medium were dried for 2 hr in a 28° incubator before inoculation. This removed surface moisture and helped to prevent spreading of the bacteria in the algal inoculum. Three small fragments (pin-head size) of inoculum were then placed equidistant apart on the agar surface, and about 25 mm. from the edge of the dish. To help further in preventing the spread of bacteria during inoculation it was found advantageous to inoculate by inverting the plate, and to bring the inoculum up from beneath, so that the surface was touched very lightly, and 'squashing' of the agar was thus minimized.

The inverted plates were incubated under continuous illumination at 25–30° until

hormogone migration occurred. Advantage was then taken of the fact that some hormogonia, devoid of the mucilaginous sheaths characteristic of older trichomes, had glided sufficiently far to free themselves from epiphytic bacteria. Hormogonia which glided slowly, or moved only a short distance from the inoculum, were followed by bacteria, from which they were unable to free themselves (Pl. 2, fig. 4, 5, 6). The next stage was the selection of uncontaminated hormogonia by micromanipulation.

Initially, clean hormogonia were selected under the phase-contrast microscope, picked up from the agar surface by a microloop, and transferred to fresh plates of the same agar medium. They invariably failed to grow; possible reasons for this will be discussed later. To eliminate direct manipulation of the hormogonia, uncontaminated ones were selected and a small square of clean agar circumscribed about them by means of a microprobe. The size of the squares varied according to the degree of spreading of the contaminating bacteria, but was usually about 1–2 mm. square (Pl. 2, fig. 6). By using an iridotomy knife (a very fine scalpel with a 5 mm. curved blade) the small agar blocks were then dissected out under a stereo microscope, and inverted onto fresh agar. Again the hormogonia failed to grow. Next, the blocks were transferred to the small cups with liquid medium, as described previously for preliminary purification, but again without success. Finally, blocks were prepared as before, and transferred to tubes of silica gel medium. After incubation under continuous illumination, algal growth appeared in most of the tubes after 1–2 weeks.

One of the soil isolates was an Anabaena species, whose hormogonia glided over the agar very slowly, and thus did not free themselves from contaminating bacteria. At a certain stage in its developmental cycle the organism produced akinetes (spores), which became detached from the parent filament. By using a microloop with a diameter slightly greater than that of the akinetes (about 20 μ), the latter were transferred to silica gel medium, on which they germinated after 4 to 6 weeks. Before transfer they were freed from bacteria by gently gliding the microloop over clean areas of the original plate. Under phase contrast there was no problem in deciding whether or not all the bacteria had been removed.

Another Anabaena species was isolated from vegetative material. The bacteria-free terminal regions of some long radiating filaments were cut off with a microprobe, then freed from bacteria by gliding them over a clean agar surface with a microloop. They were then transferred to silica gel tubes by the agar block method described previously, and growth became visible after 3 weeks. However, only a small proportion of the filaments thus isolated grew.

As a test for contamination, samples of growth from the silica tubes were streaked on the algal agar medium, incubated under light for 5 days, and examined under phase contrast. As a further test, peptone + yeast-extract (both agar and liquid media), Jensen dextrin agar for Azotobacter, and Brewer medium for anaerobes, were inoculated with algal samples. After incubation at 28° and 37° for 5 days, the plates were examined under phase contrast. These tests revealed a few cases of contamination, probable reasons for which will be discussed later.

RESULTS

Thirteen species, isolated from a Black Earth soil, were obtained in axenic culture by the methods described. They have been tentatively identified as three species of

the genus *Nostoc*, two each of *Calothrix*, *Scytonema*, and *Microchaete*, one each of *Wollea* and *Oscillatoria* (all isolated from hormogonia), one species of *Anabaena* isolated from akinetes, and another species of *Anabaena* derived from filament fragments. In addition, axenic cultures of *Nostoc muscorum* and *Oscillatoria amoena*, from the Departmental culture collection, were obtained from hormogonia. Axenic cultures of the endophytes of the cycads *Macrozamia lucida* (Linnaeus) Johnson, *Encephalartos altensteinii* Lehm., and *Bowenia serrulata* Bull, and the endophyte of the angiosperm *Gunnera chilensis* Endl., were also derived from hormogonia.

The anatomy of the coralloid roots of *Macrozamia communis* L. Johnson was described in detail by Wittmann, Bergersen & Kennedy (1965), but they were unable to obtain an axenic culture of the agal endophyte. The present report appears to be the first time that the endophyte of *Bowenia serrulata* has been isolated. This genus is endemic to Australia (Johnson, 1959). Spratt (1915) examined the roots of *B. serrulata* plants growing in Kew Gardens, but she found no alga-containing coralloid roots.

The endophytes of the cycads and of the genus *Gunnera* have been variously identified as species of *Nostoc* or *Anabaena* (Spratt, 1915; Winter, 1935; Schaede, 1951; Douin, 1953; Watanabe & Kiyohara, 1963). The filamentous characteristic of aggregating into definite colonies in culture, as observed in the present study (Pl. 2, fig. 4), would suggest that the endophytes belong to the genus *Nostoc*. The filaments of *Anabaena* do not form such colonies (Prescott, 1964).

The four endophytes and twelve of the thirteen soil isolates have all been maintained through numerous sub-cultures in nitrogen-free liquid medium. This suggests they are capable of fixing nitrogen. The species of *Oscillatoria* requires combined nitrogen for growth.

DISCUSSION

The origin of the occasional contamination of cultures developed from initially pure material seems to lie in the transfer of the agar blocks. In some cases the hormogonia migrated only a small distance (a few mm.) from the parent inoculum, so that unless the agar blocks came away cleanly during removal, their edges could become contaminated by bacteria on the adjacent agar surface. This problem was minimized by pouring a thin layer of medium over a basal layer which had been allowed to set for several hours; the thin layer was more easily removed, since it eliminated breaking of the small blocks.

Migration of hormogonia on the silica gel medium was superior to that on agar, but its use for primary isolation purposes was impractical, because of its characteristic water of syneresis, which allowed bacteria to spread over the entire plate. For this reason, the relatively hard 1·5% agar (Difco) was used. Hormogonium production and migration seemed to be better on Oxoid Ion agar No. 2, as compared with that on the Difco product; it was therefore used throughout the latter part of the work. The very firm gel produced at 1·3% (w/v) Ion agar No. 2 did not inhibit migration, and greatly facilitated the removal of the agar blocks.

It is not clear why transferred hormogonia did not grow on agar media, but grew very successfully on silica gel. This phenomenon has also been encountered by Professor G. E. Fogg (personal communication). It seemed possible that insufficient free moisture is available from firm agar gels, but growth could not be induced even when the agar concentration was decreased to 0·3%, which gave a very soft medium

just sufficiently firm to support the small agar blocks. That agar itself is toxic seems very unlikely, since hormogonia migrating on agar plates develop into colonies quite satisfactorily when not transferred, and cultures transferred from silica gel to agar develop normally, provided inocula of macroscopic size are used. The possibility that developing hormogonia require some diffusible substance from the parent colony also seems unlikely, since any such factor would be eliminated when the agar blocks are transferred to silica gel.

The failure of hormogonia to grow when transferred directly in microloops is possibly due to osmotic damage caused by rapid drying of the moisture film on the loop during the transfer. This supposition is supported by the fact that hormogonia which are floated in a water film across the agar may survive and even multiply, for several days; but they too eventually die. Perhaps the hormogonia suffer mechanical damage during manipulation, since they are observed to disintegrate within a few hours to several days of such treatment. It may be significant that akinetes were not damaged during transfer, possibly as a result of their thickened walls, and consequent ability to withstand desiccation and mechanical injury.

There was never any doubt about the purity of the migrating hormogonia. Under the phase-contrast microscope, bacteria could be seen along the slime tracks produced by the hormogonia, some of which eventually out-stripped the bacteria. Slow moving hormogonia were unable to free themselves from bacteria, which remained as a mantle about the algal filament (Pl. 2, fig. 5). The outstanding advantage of the method described is that, by direct microscopic selection of pure algal material, the factor of chance is practically eliminated, and arises only occasionally through contamination during the second stage of the transfer process. Such contamination is readily detected. A further advantage lies in the time factor, since axenic cultures can be developed within a few weeks, and the large number of successive transfers involved in other techniques is eliminated.

We wish to express sincere thanks to Mr H. C. Caulfield, Curator of the Botanic Gardens, Brisbane, for providing material of *Gunnera chilensis* and *Encephalartos altensteinii*; to Mr P. Gordon, Forestry Officer at Byfield, Q., for *Bowenia serrulata*, and to Dr A. W. Moore, C.S.I.R.O. Cunningham Laboratory, for *Macrozamia lucida*. Thanks are due to Mr G. Jurott, Dept. of Clinical Photography, University of Queensland, for the photographic work. The work was supported by a grant from the Rural Credits Department of the Reserve Bank of Australia.

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Section of a coralloid root of the cycad *Bowenia serrulata*, 25 days after inoculation onto silica gel, showing the 'algal zone' (AZ), and algal colonies (AC) developing around the periphery. The Petri dish was incubated at 25–30° under continuous fluorescent illumination of 800 lux intensity. × 10.

Fig. 2. The coralloid root section (RS) seen in fig. 1, 63 days after inoculation, showing the development of extensive algal growth (A). The piece from the top left corner had been removed as inoculum for purification. × 5.

Fig. 3. Stem tissue (S) containing the endophyte of *Gunnera chilensis*, from which hormogonia have migrated on the silica gel medium, to produce the radial arrangement of algal colonies (AC); 25 days after inoculation, incubated at 25–30° under continuous fluorescent illumination of 800 lux intensity. × 10.

PLATE 2

Fig. 4. The endophyte of *Bowenia serrulata*, growing on mineral salts agar. Note the numerous gliding hormogonia (H), some of which have developed into filaments forming well-defined colonies (C). × 100.

Fig. 5. Hormogonium of a Nostoc soil isolate, gliding on mineral salts agar. This one had glided only a short distance and had not freed itself of contaminating bacteria (B), which remain as an enveloping mantle. Phase contrast. × 250.

Fig. 6. Two uncontaminated hormogonia of the endophyte of *Gunnera chilensis*, gliding on mineral salts agar. Note the 'slime track' (ST) left on the agar surface. The bordering lines (BL) were marked out with a microprobe, attached to the micromanipulator, and the agar block so defined is ready for transfer. Phase contrast. × 100.

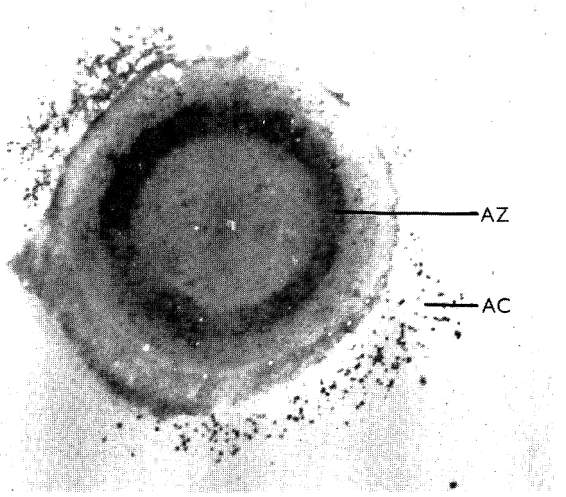

Fig. 1

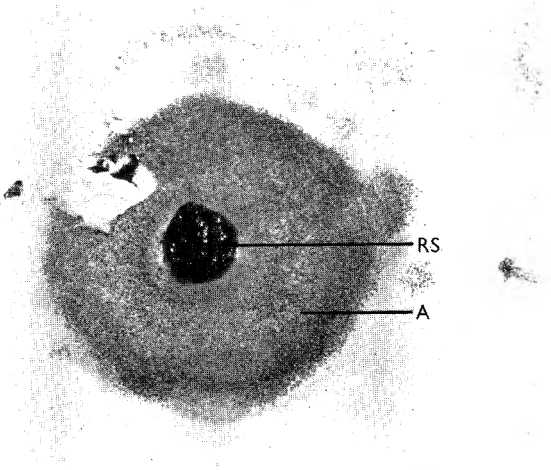

Fig. 2

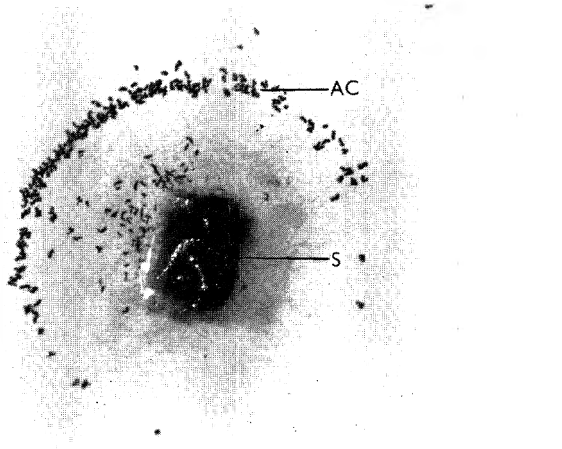

Fig. 3

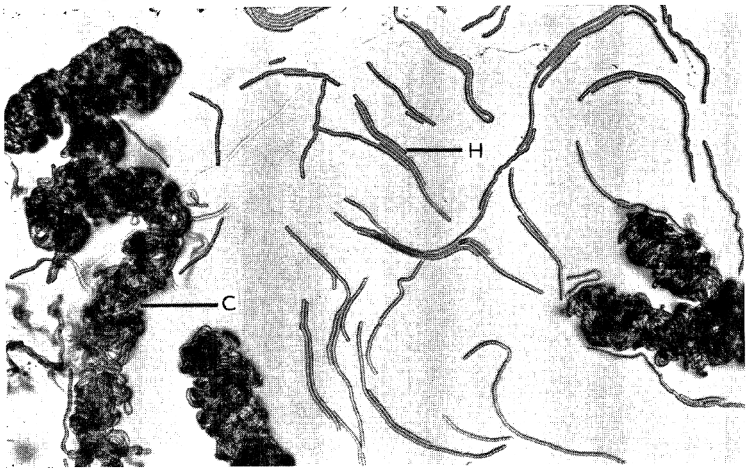

Fig. 4

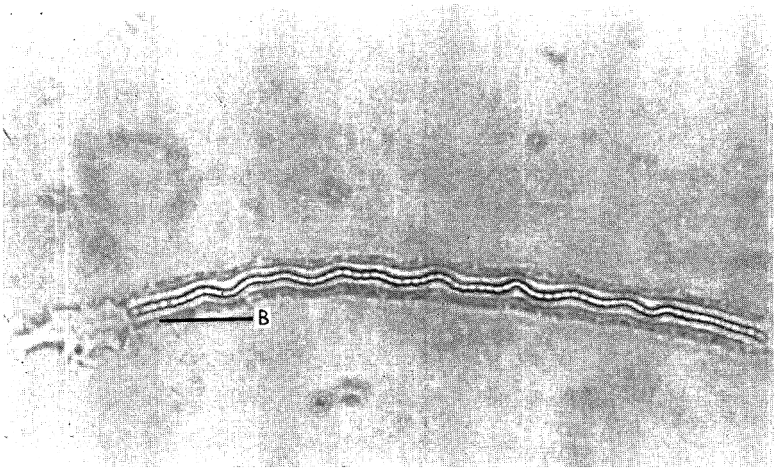

Fig. 5

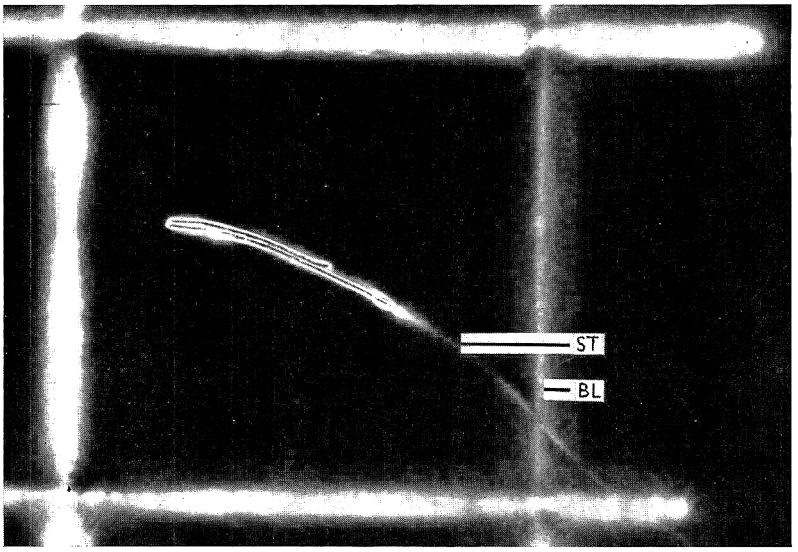

Fig. 6

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Thymidine Kinase: Evidence for its Absence from *Neurospora crassa* and Some Other Micro-organisms, and the Relevance of This to the Specific Labelling of Deoxyribonucleic Acid

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SUMMARY

Thymidine kinase (EC 2.7.1.21), an enzyme that catalyses the phosphorylation of thymidine to thymidine-5′-phosphate in the presence of adenosine triphosphate and Mg^{2+}, was not detected in cell-free extracts of *Neurospora crassa*, *Aspergillus nidulans*, *Saccharomyces cerevisiae* or *Euglena gracilis*. Deoxyribonucleic acid was not specifically labelled in *Neurospora crassa* grown in the presence of [^{14}C]thymidine, [^{14}C]thymidine monophosphate or [^{14}C]thymidine triphosphate. Instead, the isotope was incorporated into both types of nucleic acid in a ratio which approximated the mole ratio of ribonucleic acid to deoxyribonucleic acid. It is considered that the absence of thymidine kinase prevents the specific incorporation of thymidine into deoxyribonucleic acid in intact cells of *Neurospora crassa*.

INTRODUCTION

The incorporation by micro-organisms of [^{3}H]- and [^{14}C]-labelled thymidine has often been used as a simple and reliable technique for the specific labelling of newly synthesized deoxyribonucleic acid (DNA) (Evans, 1966). This procedure has not been successfully applied to the ascomycete *Neurospora crassa*. Neither thymine- nor thymidine-requiring mutants of *N. crassa* have been reported (*Neurospora* Stock List, Fungal Genetics Stock Centre, Dartmouth College, U.S.A.) and thymidine will not support the growth of pyrimidine-requiring *N. crassa* mutants (Fink & Fink, 1961) which otherwise grow in minimal medium when supplemented with uracil, uridine or cytidine (Loring & Pierce, 1944; Loring, Hammell, Levy & Bortner, 1952; Chakraborty & Loring, 1960). Although the tracer from labelled thymidine is incorporated by *N. crassa* into nucleic acids, the label is not confined to DNA (Chakraborty & Loring, 1960; Fink & Fink, 1961, 1962 *a*, *b*). The fact that *Neurospora crassa* does not specifically incorporate exogenous thymidine led Chakraborty & Loring (1960) and Fink & Fink (1962 *a*) to postulate that thymidine kinase is absent from this fungus. However, the phenomenon might also be explained by active degradation of added thymidine (Adelstein & Lyman, 1968) or in terms of membrane permeability effects (see Lieberman, Kornberg & Simms, 1955). Radioautographic evidence for other fungi and some algae suggests that other micro-organisms may also be deficient in thymidine kinase (Cleaver, 1967). In labelling experiments, [^{3}H]thymidine was not

detected in the nuclei of two pathogenic fungi, *Puccinia graminis* var. *tritici* (Bhattacharya & Shaw, 1967) and *Uromyces phaseoli* (Staples & Ledbetter, 1960), and the label was found in both ribonucleic acid (RNA) and DNA of *Euglena gracilis* (Sagan, 1965), *Spirogyra grevilleana* (Meyer, 1966) and *Schizosaccharomyces pombe* (Mitchison, 1963).

In the present work, cell-free extracts of several micro-organisms were studied for the presence of the enzyme thymidine kinase (ATP: thymidine 5′-phosphotransferase; EC2.7.1.21) (Okazaki & Kornberg, 1964). Also, thymidine and thymidine nucleotides were supplied to cultures of *Neurospora crassa* in an attempt to label specifically the DNA.

METHODS

Organisms. The micro-organisms used were *Escherichia coli* strains B and B-185, *Aspergillus nidulans* BI-1, *Saccharomyces cerevisiae* (DELFT 1171), and *Neurospora crassa* strains STA 4 (wild-type) and *pyr*-3 (1298). Strain 1298 of *N. crassa* requires a pyrimidine supplement, and is able to grow in a medium containing uracil, uridine or cytidine (Loring *et al.* 1952).

A frozen preparation of broken cells of *Euglena gracilis* was obtained from Dr N. S. Scott (CSIRO Division of Food Preservation, University of Sydney, Australia).

Culture media and growth conditions. Escherichia coli was grown in Hershey's nutrient broth (Chase & Doermann, 1958) with extra glucose 0·5 (g./l.). *Neurospora crassa* was grown in the minimal N medium of Vogel (1956). The carbon source in solid agar media (2%, w/v, Bactoagar) was 1% (w/v) sucrose and 1% (v/v) glycerol; in liquid cultures it was 2% (w/v) sucrose. The media for strain 1298 were supplemented with uracil (Nutritional Biochemicals Corp., U.S.A.) (0·4 mg./ml.), except in labelling experiments when 0·05 mg./ml. was used. In one experiment, the minimal medium was supplemented with 0·2% yeast extract. *Aspergillus nidulans* was grown in minimal medium (Cove, 1966) containing 0·1% yeast extract. *Saccharomyces cerevisiae* was grown in YEPD medium which contains yeast extract (Manney, 1964).

The following Difco products (Difco Laboratories, Michigan, U.S.A.) were used in the preparation of media: nutrient broth, Bacto-peptone, yeast extract and Bacto-agar. All media were autoclaved at 120° for 10 to 15 min. except in the case of labelling experiments to be described later. The micro-organisms were grown in 100 ml. medium in 500 ml. conical flasks. Inocula of *Escherichia coli* and *Saccharomyces cerevisiae* were actively growing liquid cultures. Suspensions of conidia in water, prepared from 5- to 10-day cultures of *Neurospora crassa* and *Aspergillus nidulans*, were used as inocula. Liquid cultures were aerated for 14 to 20 hr at 28° to 30°; at 37° for *E. coli*.

Preparation of thymidine kinase from Escherichia coli. A partially purified enzyme was prepared from *Escherichia coli* B-185 according to the method of Okazaki & Kornberg (1964). The enzyme fraction used was fraction 5 of their procedure ('fraction 5 thymidine kinase').

Reagents. The labelled materials used were obtained from the following sources: [2-^{14}C]thymidine (35·9 mc./m-mole) and [2-^{14}C]uridine (36·7 mc./m-mole) from the Radiochemical Centre, Amersham, Buckinghamshire, U.K., and [2-^{14}C]thymidine triphosphate (50 mc./m-mole) from Schwartz BioResearch Inc., Orangeburg, New York, U.S.A.

[2-^{14}C]Thymidine monophosphate was prepared enzymically from [2-^{14}C]thymidine with the fraction 5 thymidine kinase as indicated by Okazaki & Kornberg (1964). The product was isolated by low-voltage paper electrophoresis on Whatman 3MM paper with sodium citrate buffer (0·05 M; pH 3·4), and eluted from the paper with water (Dent, 1947).

[^{14}C]DNA was extracted by the method of Marmur (1961) from the chemoautotrophic bacterium *Nitrosomonas europaea* grown in medium containing [^{14}C]carbonate (36·4 mc./m-mole; Radiochemical Centre, Amersham, U.K.).

Unlabelled di- and tri-phosphates of adenosine (ADP, ATP) and mono- and triphosphates of thymidine (TMP, TTP) were bought from Calbiochem (California, U.S.A.). Thymine and thymidine (dT) were obtained from the Sigma Chemical Co. (St Louis, Mo., U.S.A.). All other chemicals were of analytical reagent grade.

Preparation of cell-free extracts. All operations following the collection of organisms were done at 0° to 4°. With the exceptions described in the Results section, the buffer used in these experiments was tris-HCl (2-amino-2-hydroxymethylpropane-1,3-diol chloride; 0·05 M, pH 7·5). Organisms were collected by centrifugation at 7000 to 15,000 *g* for 2 to 10 min., washed in buffer once or twice and resuspended in 2 to 7 ml. buffer/g. wet weight organism. The organisms were extracted by ultrasonic disruption for 2 to 5 min. (MSE 20 kc. Ultrasonic Disintegrator fitted with a 0·9 cm. titanium probe). Each crude homogenate was centrifuged at 5000 *g* for 10 min. to remove cell debris. Supernatant fractions were used for all enzyme assays. After precipitation with 10% (w/v) trichloroacetic acid, protein concentrations were determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

Measurement of thymidine kinase activity. The basic reaction mixture for thymidine kinase determinations was essentially that described by Okazaki & Kornberg (1964); the main difference was the inclusion of an ATP regenerating system. The reaction volume (0·07 ml.) contained buffer (tris-HCl, pH 7·5; 5·0 μmoles), bovine serum albumin (20 μg.; Commonwealth Serum Laboratories, Australia), ATP (0·15 μmole), $MgCl_2$ (0·50 μmole), creatine phosphate (0·7 μmole), creatine phosphokinase (4 μg.; Calbiochem), thymidine (0·012 or 0·017 μmole; specific activity $5{\cdot}6 \times 10^6$ or $1{\cdot}8 \times 10^7$ counts/min./μmole) and cell-free extract (0·01 ml. containing 0·5 μmole buffer and 0·02 to 0·40 mg. protein). Variations from this mixture are recorded in the relevant sections below. Extracts heated in boiling water for 2 min., as well as fraction 5 thymidine kinase, were used in separate control incubations. After 30 min. incubation at 37° each reaction was stopped by immersing the tubes in boiling water for 1·5 min. The precipitate was removed after centrifugation at 2000 *g*, and duplicate 0·025 ml. samples were analysed by low-voltage paper electrophoresis, together with added marker compounds thymidine, TMP and TTP. Whatman 3MM paper and 0·10 M-sodium citrate buffer (pH 3·4) were used. Marker areas, located with short wavelength ultraviolet radiation (Mineralight model SL 2537), were cut out and the quantity of isotope in each measured in a liquid scintillation spectrometer (a model 3002 Tricarb Liquid Scintillation Spectrometer, Packard Instrument Co. Inc., Illinois, U.S.A.; or a model 6851 Unilux liquid scintillation system, Nuclear Chicago Corp., Illinois, U.S.A.). The scintillation fluid contained 0·20 g. POPOP (1,4-bis-2-(5-phenyloxazolyl)-benzene)/l. and 3·0 g. PPO (2,5-diphenyloxazole)/l. in toluene. Most samples were counted for 20 min. The quantity of [2-^{14}C]thymine in the reaction mixture after incubation was determined by liquid scintillation counting. The thymine and thymidine

in 0·01 ml. samples of the reaction mixtures were separated by ascending paper chromatography in 0·25 N-formic acid on Whatman 1 paper (Shapiro, Eigner & Greenberg, 1965).

Thymidine kinase activity is expressed in the unit μm-mole TMP produced in 30 min. at 37° per assay. All tabulated data have been corrected by subtraction of the background quantity of label corresponding to TMP in heated-extract control incubations.

Labelling of intact Neurospora crassa. Samples (2·5 ml.) of Vogel's minimal N medium, supplemented with 0·15 mg. uracil and one of the [^{14}C]-labelled compounds, were individually sterilized by passing through a membrane filter (13 mm., 0·22 μ; Millipore Corp., Mass., U.S.A.). This procedure was adopted because autoclaving was associated with dephosphorylation of labelled nucleotides. Conidia from 6-day cultures of *Neurospora crassa*, STA 4 and 1298, were suspended in minimal medium. Hyphal fragments were removed from this inoculum by filtration through fine nylon mesh. Approximately $2{\cdot}5 \times 10^8$ conidia in 0·5 ml. were added to each incubation medium in an 18 × 150 mm. sterile test tube. The cultures were vigorously agitated by a wrist-action shaker for 16–18 hr at 28° to 30°. No evidence for bacterial contamination after incubation was detected by the naked eye, by phase-contrast microscopy, or after small samples of some cultures had been streaked onto solid nutrient medium (Hershey's nutrient broth; Chase & Doermann, 1958).

The cultures were collected by centrifugation at 5000 *g* for 5 min. at 4°. Except where otherwise stated, all subsequent operations were done at 0° to 4°. After washing twice with 3 ml. water, the organisms were suspended in a total volume of 2·5 ml. of water containing 0·10 μmole DNA-phosphorus as calf thymus DNA. They were disrupted ultrasonically for 4 to 6 min. and centrifuged at 5000 to 7000 *g* for 5 min. Each pellet was washed with 0·5 ml. water and the washing was bulked with the respective supernatant fraction. The supernatant fluids were then fractionated according to a variation of the Schmidt–Thannhauser procedure (Munro & Fleck, 1966), to yield the following fractions: perchloric acid-soluble; acid-insoluble but alkali-hydrolysable (RNA); acid-insoluble and alkali-non-hydrolysable (DNA). The lipid extraction step was omitted and the period of hydrolysis of RNA at 37° was from 2 to 4 hr. Samples of each fraction, dried on Whatman 3 MM paper, were counted in a liquid scintillation spectrometer for 40 min. Nucleic acid bases were released by hydrolysis of further samples with 99% formic acid in sealed Pyrex-glass tubes at 175° for 30 min. (Wyatt, 1951). Marker bases were derived from either uracil + calf thymus DNA or thymine + yeast RNA (Schwartz BioResearch Inc.). These were included with the sample being hydrolysed. This also enabled a check to be made that each hydrolysis was complete. The dried hydrolysates, dissolved in 0·1 N-HCl, were separated by ascending paper chromatography on Whatman 1 paper in *iso*-propanol + water + conc. HCl (65 + 18·4 + 16·6 by vol.; Wyatt, 1951). After detection with short-wavelength ultraviolet radiation, areas containing nucleic acid bases were cut out and counted in a liquid scintillation system. The possibility that label was present in other areas of the chromatograms was tested by dividing replicate chromatograms into 0·25 in. sections and counting each section in sequence. Moreover, when compared with a photographic print made with ultraviolet radiation (Markham & Smith, 1949) the resulting pattern of label corresponded closely with the ultraviolet radiation-absorbing material.

RESULTS

Thymidine kinase activity in cell-free extracts

Except for extracts of *Escherichia coli* B, significant thymidine kinase activity was not detected in extracts of any of the micro-organisms tested (Table 1). When cell-free extracts of *E. coli* B were mixed in turn with the other extracts, the resultant activity was greatly diminished (Table 1). This suggested the presence of an active inhibitor, a thymidine degradative activity, or a TMP phosphatase activity. Most of the ATP was still present at the completion of each incubation, suggesting that this result was not due entirely to the presence of an ATPase. However, when the mixed incubations were repeated with the fraction 5 thymidine kinase instead of a cell-free extract of *E. coli*, the inhibition of the thymidine kinase activity was markedly less (Table 1).

Table 1. *Thymidine kinase activities in cell-free extracts of various micro-organisms, each assayed separately and when mixed with enzyme fractions of Escherichia coli*

	Cell-free extract	Thymidine kinase activity		
Organism	Protein concn. (mg./assay)	Extract alone	Combined with cell-free extract of *E. coli*†	Combined with fraction 5 thymidine kinase
Control	—	0·000	0·565	0·943
N. crassa STA 4	0·11	0·004	0·088	0·634
N. crassa STA 4*	0·10	0·001	0·146	0·598
A. nidulans	0·10	0·001	0·183	0·685
S. cerevisiae	0·40	0·004	0·035	0·748
E. gracilis	0·02	0·001	0·325	0·872

* Organisms grown in minimal medium supplemented with yeast extract. Assays as described in Methods. Values mean of four determinations: μm-mole TMP produced in 30 min./assay.

† Protein added in *E. coli* crude extract was 0·10 mg./assay.

When the time-course of the reaction was followed (Fig. 1 *a*), the reaction catalysed by the *Escherichia coli* extract was non-linear over a 30 min. period and the enzyme activity was significantly depressed when *E. coli* extract was mixed with that of *Saccharomyces cerevisiae*. In contrast, corresponding reactions with the fraction 5 thymidine kinase yielded curves which closely correspond (Fig. 1 *b*). When the concentration of ATP was increased in reaction mixtures containing extracts of both *E. coli* and *S. cerevisiae* (Table 2), the final yield of TMP was increased. These data (Table 2) also showed that little of the initial thymidine remained after a 30 min. incubation, and that the rate of degradation of thymidine to thymine was decreased in the presence of greater concentrations of ATP. It was also observed that when an *E. coli* extract alone was used with normal amounts of ATP (0·15 μmole/assay), practically all the initial thymidine was degraded. In incubation mixtures which did not include a crude extract of *E. coli*, the degradation of thymidine never exceeded 10% of the amount originally added. These data have been collectively interpreted to indicate that cell-free extracts of *E. coli* degrade thymidine, and that this effect is decreased by high concentrations of ATP. Addition of other extracts may promote the hydrolysis of ATP, resulting in an effective increase in this activity. For this reason,

fraction 5 thymidine kinase was used instead of *E. coli* cell-free extract in subsequent control incubations.

No activity was detected after extraction of *Neurospora crassa* in a ground-glass homogeniser. In contrast, TMP kinase activity was readily measured in extracts prepared by homogenization and ultrasonic disintegration. Thymidine kinase activity was not detected even when two strains of *N. crassa* (1298, STA 4) were extracted

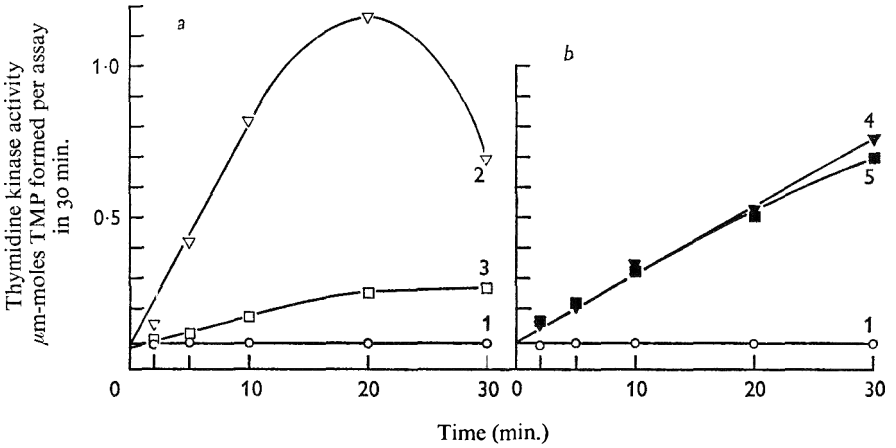

Fig. 1. The effect of cell-free extracts of *Saccharomyces cerevisiae* on *Escherichia coli* thymidine kinase. Assays as described in Methods. Incubations terminated at specified times after adding the enzyme fractions. *a*, Extract of *S. cerevisiae* assayed alone and when mixed with cell-free extract of *E. coli*. *b*, Extract of *S. cerevisiae* assayed alone and when mixed with fraction 5 thymidine kinase: 1, Cell-free extract of *S. cerevisiae*; 2, cell-free extract of *E. coli* B; 3, mixture of 1 and 2; 4, fraction 5 thymidine kinase; 5, mixture of 1 and 4.

Table 2. *Influence of initial ATP concentration on the amount of TMP synthesized, and on thymidine degradation to thymine by cell-free extract of Escherichia coli mixed with that of Saccharomyces cerevisiae*

Assay conditions as described in Methods (initial concentration of thymidine constant at 11·7 μm-mole/assay), except that the initial quantity of ATP per assay was increased to the value listed in the table. The listed quantities of TMP, thymidine and thymine are in units of μm-mole of compound present per assay after a 30 min. incubation.

Cell-free extract	Initial quantity of ATP (μmole)	TMP produced	Thymidine remaining	Thymine released
E. coli B	1·35	0·95	—	—
E. coli + *S. cerevisiae*	0·15	0·10	1·8	9·8
E. coli + *S. cerevisiae*	0·75	0·56	3·1	8·1
E. coli + *S. cerevisiae*	1·35	0·65	4·2	6·8

and assayed in the presence of each of three buffers (tris-HCl, potassium phosphate, potassium phosphate + 10^{-4} M-EDTA; all 0·05 M, pH 7·5). It was calculated from these data (Student's *t* test) that at the 95% confidence level this set of assays would have detected as significant a mean activity in excess of that for the control assays equal to 0·010 μm-mole TMP produced in 30 min. per reaction mixture. Allowing for the range in the quantity of protein in the individual assay reactions, this rate corre-

sponds to a minimal detectable specific activity of 0·24 to 0·44 μm-mole TMP synthesized/mg. protein in 30 min.

When mixtures of fraction 5 thymidine kinase and cell-free extracts of *Neurospora crassa* STA4 were tested for activity, with and without an ATP regenerating system (creatine phosphate + creatine phosphokinase), the results showed that the apparent inhibition of the thymidine kinase activity by the crude extract was completely reversed in the presence of an adequate amount of ATP (Table 3). It was therefore concluded that the non-detection of a thymidine kinase activity in extracts of *N. crassa* did not result from the presence of an inhibitor, a TMP phosphatase activity, an ATPase or a thymidine degradative enzyme.

Table 3. *Activity of fraction 5 thymidine kinase with and without a cell-free extract of Neurospora crassa and an ATP-regenerating system*

Assays as described in Methods. Buffer was potassium phosphate (0·05 M; pH 7·5); 0·05 mg. protein in *N. crassa* extract per assay. The ATP regenerating system was creatine phosphokinase + creatine phosphate (see Methods). Units are μm-mole TMP produced in 30 min./assay.

Fraction assayed	No ATP regenerating system	+ ATP regenerating system
Extract of *N. crassa* STA 4	0·00	0·00
Fraction 5 thymidine kinase	0·83	0·87
N. crassa + fraction 5 thymidine kinase	0·53	0·90

Labelling of intact cells of Neurospora crassa

When *Neurospora crassa* strains 1298 and STA4 were grown in the presence of [^{14}C]thymidine, the isotope was recovered in both the RNA and DNA fractions (Table 4). Had a specific thymidine kinase been active, it is reasonable to expect that the greater part of the label would have been in the DNA, instead of the RNA as observed (ratio RNA:DNA, Table 4). Several lines of evidence show that this accumulation of label in the RNA fraction cannot be explained simply by hydrolysis of DNA during the fractionation: (1) In an experiment with non-labelled *N. crassa* to which [^{14}C]DNA had been added before extraction and fractionation, less than 6% of the acid-insoluble labelled material was hydrolysed by the alkaline treatment. (2) In RNA fractions from labelled organisms, the tracer was recovered in uracil but not in thymine (Table 5). (3) The ratio of labelled RNA:labelled DNA in the cultures (5 to 16, Table 4) agreed with the value of 8 determined previously (Chakraborty & Loring, 1960).

Similar distributions of the label between RNA and DNA were observed regardless of whether *Neurospora crassa* was grown in the presence of [2-^{14}C]thymidine, TMP, TTP or uridine (Table 4). TTP was incorporated with relatively low efficiency compared with the other compounds. The similarity of the labelling patterns in cultures containing thymidine derivatives and in those supplemented with uridine strongly suggested that the four labelled compounds tested were incorporated by way of a common metabolic intermediate. This might be uridine or a derivative (Fink & Fink, 1962 *b*). This hypothesis was supported by the data of Table 5, which show that the tracer was recovered in the three major pyrimidine bases. The amount of label in the purine bases (adenine, guanine) was negligible, never exceeding 5% of the total recovered isotope. Cytosine and thymine derived by hydrolysis of each DNA fraction, were labelled with similar specific activities. This followed from the fact that when the

mole ratio (expressed as mole % guanine+cytosine) was calculated for DNA from the proportions of labelled bases, the figure obtained (52·1 %) was in agreement with a previously reported value for *N. crassa* (52 to 55%; Storck, 1966). The estimated value was calculated from the data of Table 5, excluding those for the uridine-labelled material. The quantity of labelled cytosine in DNA was obtained by subtraction of the cytosine estimated to be derived from RNA contaminating the DNA fractions. The RNA-cytosine was estimated from the amount of uracil in the DNA fractions, assuming a value of 1·025 for the ratio cytosine:uracil in *N. crassa* RNA (Storck,

Table 4. *Neurospora crassa: distribution of* [^{14}C] *among fractions derived from intact cells grown in medium containing labelled pyrimidine nucleosides and nucleotides*

Expt. no.	Strain of *N. crassa*	[^{14}C]-substrate: Compound	[^{14}C]-substrate: Quantity added (μm-mole)	[^{14}C]-substrate: Proportion incorporated	Proportion of label added initially: Acid-soluble	Proportion of label added initially: RNA	Proportion of label added initially: DNA	Ratio of label in RNA: label in DNA
1	1298	dT	3·83	0·64	0·306	0·220	0·032	6·96
		TMP	3·78	0·80	0·311	0·334	0·065	5·14
2	1298	dT	3·92	0·69	0·114	0·422	0·044	9·69
		TMP	3·78	0·78	0·136	0·499	0·049	10·22
		TTP	4·00	0·28	0·084	0·164	0·013	12·88
		Uridine	5·44	0·54	0·056	0·359	0·023	15·92
3	1298	dT	3·92	0·83	0·214	0·444	0·052	8·57
		TMP	3·78	0·89	0·219	0·461	0·059	7·75
		TTP	4·00	0·46	0·103	0·196	0·025	7·92
		Uridine	5·44	0·87	0·166	0·526	0·052	10·14
4	STA 4	dT	3·83	0·72	0·199	0·421	0·045	9·41

Table 5. *Distribution of* [^{14}C] *among the pyrimidine bases within RNA and DNA fractions prepared from labelled Neurospora crassa*

The data were obtained, as described in Methods, by hydrolysis and fractionation of RNA and DNA obtained from organisms supplied with the specified labelled substrate. The RNA and DNA fractions listed correspond to those of Expts. 2 and 4 of Table 4. Values are expressed as proportion of label recovered from each chromatogram.

N. crassa strain	Labelled supplement	RNA: Thymine	RNA: Uracil	RNA: Cytosine	DNA: Thymine	DNA: Uracil	DNA: Cytosine
STA 4	dT	0·03	0·45	0·48	0·29	0·16	0·51
1298	dT	0·03	0·50	0·41	0·36	0·11	0·49
1298	TMP	0·04	0·47	0·42	0·34	0·13	0·50
1298	TTP	0·03	0·53	0·37	0·37	0·11	0·49
1298	Uridine	0·03	0·46	0·46	0·38	0·09	0·50

1965). This agreement between the estimated and reported values of the mole ratio rendered it unlikely that even a small proportion of the thymidine was incorporated via a direct metabolic pathway into DNA (presumably including thymidine kinase), since this would have resulted in a marked decrease in the estimated mole ratio for the

DNA fractions. The data of Tables 4 and 5 agree with those previously obtained from similar labelling experiments with *N. crassa* (Chakraborty & Loring, 1960; Fink & Fink, 1961; 1962 *a*, *b*).

Although these experiments showed that label from [2-^{14}C]thymidine, TMP and TTP entered *Neurospora crassa* and was incorporated into nucleic acids, the distribution of the isotope revealed that it was not incorporated directly into DNA. These data are therefore consistent with the postulate that *N. crassa* lacks a specific thymidine kinase.

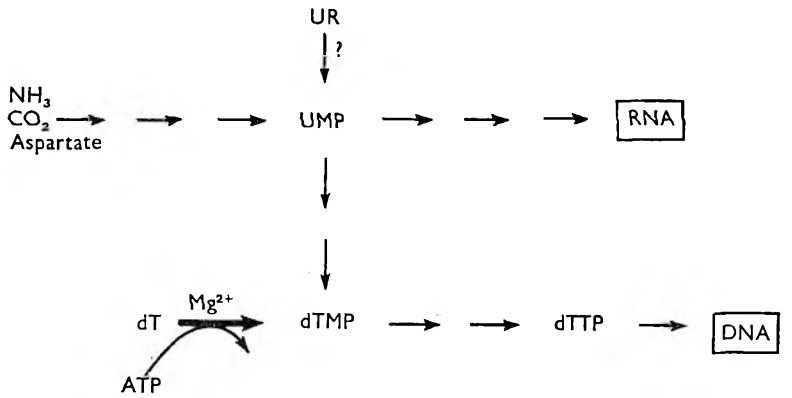

Fig. 2. Metabolic sequence for the synthesis of thymine residues of DNA in *Escherichia coli* (derived from Okazaki & Kornberg, 1964).

DISCUSSION

The metabolic relationship between the *de novo* and 'salvage' pathways of thymidine incorporation into DNA in *Escherichia coli* is shown in Fig. 2. Indirect evidence suggests that the enzyme thymidine kinase may not be present in some micro-organisms, including *Neurospora crassa* (Chakraborty & Loring, 1960; Fink & Fink, 1961; 1962 *a*; Staples & Ledbetter, 1960; Mitchison, 1963; Sagan, 1965; Meyer, 1966; Bhattacharya & Shaw, 1967). Direct experimental evidence given in the present paper strongly suggests that thymidine kinase is not present in cell-free extracts of these micro-organisms. In the absence of thymidine kinase, the normal salvage route from thymidine to DNA cannot operate. This would explain the lack of efficient or direct incorporation of thymidine into the DNA of such micro-organisms. However, tracer was certainly incorporated into the nucleic acids of *N. crassa* when supplied as [2-^{14}C]thymidine, [2-^{14}C]TMP or [2-^{14}C]TTP, although the molar quantities were very small. In fact, a supplement of thymidine does not support growth of pyrimidine-requiring mutants of *N. crassa* in minimal medium (Fink & Fink, 1961). This implies that the incorporation of label involves a relatively inactive metabolic pathway. The incorporation of thymidine is thought to involve the removal of the 5-methyl group (Fink & Fink, 1962 *a*, *b*), leaving a uridine derivative which is then further metabolized to yield all the required pyrimidine nucleotides. This may involve the enzyme thymine 7-hydroxylase, as suggested by Abbott, Kadner & Fink (1964) and Abbott *et al.* (1967). Thymidine can be utilized as a substrate by this enzyme (Abbott *et al.* 1964).

The lack of thymidine kinase in *Neurospora crassa* can explain the observed absence of specific labelling of its DNA in the presence of labelled thymidine. TMP and TTP are believed to be intermediates in the synthesis of thymine residues of DNA (Okazaki & Kornberg, 1964). Yet, data from the intact-cell labelling experiments indicate that isotope supplied as TMP and TTP was not incorporated directly into DNA either. Since this may reflect cell-membrane impermeability to these compounds (see Loring *et al.* 1952), a method for increasing cell-membrane permeability might overcome this problem (see Buttin & Kornberg, 1966). This in turn might enable DNA to be labelled with at least some increase in specificity.

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