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Studies on the Ecology of Certain Rumen Ciliate Protozoa

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SUMMARY

The times taken by *Ophryoscolex tricoronatus* and species of the genus *Epidinium* to establish in the rumens of young animals were found to differ from those of other rumen ciliate species. The antagonistic relationships between certain rumen ciliates were examined *in vivo* and concurrent experiments were made in small vessels termed vivars which were suspended within the rumen of a sheep. A Millipore membrane separated the rumen contents from the contents of the vivars. Within these vessels it was possible to reproduce population changes first observed *in vivo* and to determine with certainty that in a population change where *Polyplastron multivesiculatum* became irreversibly dominant this had been caused by predation by Polyplastron. This organism will eliminate Epidinium, *Eudiplodinium maggii*, Eremoplastron and Ostracodinium from a population. The cause, factors affecting it and the ultimate effect on the population are discussed. It was not possible to cause a reversal of the population within a vivar suspended in sheep, but since the prey of Polyplastron still existed and since the reverse change was seen to occur in cattle, this problem still remains unsolved. There was also some evidence of antagonism between Epidinium and Ophryoscolex.

INTRODUCTION

Previous studies have shown that when mixtures of rumen ciliate populations from different animals are introduced into the rumens of ciliate-free sheep or cattle certain species often become dominant (Eadie, 1962*b*). The present work extends these observations and in particular is concerned with the relation between *Polyplastron multivesiculatum* (Dogiel & Federowa) and other rumen ciliates. Investigations have also been made, incidental to the above studies, of the time taken to establish *Ophryoscolex tricoronatus* (Dogiel) in ruminants of different species, extending previous work on the establishment of rumen ciliate populations (Eadie, 1962*a*).

The problems were investigated by the use of inoculations of chosen ciliate populations to lambs, kids and a calf; an *in vivo* method previously discussed by Eadie (1962*a*, *b*). In addition a modification of the 'vivar' technique described by Fina *et al.* (1962), when used in sheep, proved to be extremely useful. As discussed earlier (Eadie, 1962*b*) certain changes of protozoal population in lambs were irreversible, but with the aid of vivar chambers, stages of population changes could be repeatedly observed and could be compared with those seen in the animal.

METHODS

Definitions of different ciliate populations

In a previous paper (Eadie, 1962*b*) the large rumen Ophryoscolecids were grouped as type A or type B according to the organisms which tended to be found together in stable populations under natural conditions. These designations have been largely retained and the organisms concerned in this paper are listed below:

Type A organisms:
- *Polyplastron multivesiculatum* (Dogiel and Fedorowa)
- *Diploplastron affine* (Dogiel and Fedorowa)
- *Ophryoscolex tricoronatus* (Dogiel)

Type B organisms:
- *Eudiplodinium maggii* (Fiorentini)
- *Epidinium* spp.
- *Eremoplastron* spp.
- *Ostracodinium* spp.

Common species:
- *Entodinium* spp.
- *Isotricha* spp.
- *Dasytricha ruminantium* (Schuberg)

For convenience the following terminology is used through this work: (i) a 'limited' population is one in which only one or two larger organisms are present along with small *Entodinium* spp., e.g. *E. simplex*; (ii) a 'mixed' population is one, more typical of those found in nature, comprising a number of *Entodinium* spp., most probably both holotrich genera (*Isotricha*, *Dasytricha*) along with larger Ophryoscolecid organisms.

In the present work though the species are divided into type A and type B 'organisms'; a 'type A population' is defined as one in which Polyplastron is present either as the only large organism in a limited population or along with other type A organisms and the 'common species'. Thus (Polyplastron + Entodinium) and (Polyplastron + Diploplastron + Ophyroscolex + Entodinium + Isotricha + Dasytricha) are each type A populations, the former being a 'limited type A population' and the latter a 'mixed type A population'.

A population with the designation (Polyplastron + Eudiplodinium + Entodinium + Isotricha + Dasytricha) which can be produced experimentally would be termed a 'combined (A + B) population', since the presence of Polyplastron denotes a type A but the other large Ophyroscolecid is type B. Such combined populations tend to be unstable. The components of a limited population containing an organism of each type are mentioned by name.

There is no species restriction on the definition of a type B population so that any population containing one or more type B organisms, with or without the common species but excluding any type A organism, comprises a type B population.

Animals and their ciliate fauna

Young animals used for in vivo studies. Over a period of 3 years, fourteen lambs and five kids were removed from the dam and reared ciliate-free until the inoculation experiments began. Calf Z (Eadie, 1962*a*) was also used. Some of these animals were later used as the source of ciliates for vivar experiments (see below).

Adult animals and their ciliate fauna. Five cannulated sheep (93, 146, 187, 208, 300) had mixed type A populations and four (3, 209, 78, 79) mixed type B populations. One sheep in each group (208, 209) was equipped with a 2 inch rumen cannula into which the vivar apparatus could be fitted. The type B population in 78 and 79 originated in cattle and included tailed epidinia and Eremoplastron. At different periods limited ciliate populations were available from the animals listed in Table 1 and sheep 662 was maintained completely ciliate-free. A dairy cow was used to provide a mixed type B cattle ciliate population.

Diets and management. With the exception of sheep 300, which was given a pelletted barley diet, the diets and management of the sheep were as described by Eadie (1962*a*). The kids were given a lamb diet and maintained in isolation pens similar to those used for lambs. The management of calf Z has already been described. (Eadie, 1962*a*).

Table 1. *Animals with limited ciliate populations available during vivar experiments*

Animal and number		Ciliate population: Small entodinia plus: Polyplastron	Ophryoscolex	Eudiplodinium
Sheep	53	.	×	×
	95	.	.	×
	661	.	×	.
	683	×	.	.
	71	×	.	.
Goats	G 108	×	×	.
	G 110	×	.	.
	G 202	×	.	.

Procedure for in vivo studies

Fina *et al.* (1962) referred to their vivar technique as an *in vivo* method. To avoid confusion in the present work *in vivo* experiments will refer exclusively to those in which observations were made on changes within the rumen of an animal, and the vivar technique will be referred to by name.

The methods of sampling and inoculating animals without cannulae were those used previously (Eadie, 1962*a*), as was the examination procedure. In general the concentrated organisms from 500 ml. rumen fluid were used as a calf or sheep inoculum and that from 250 ml. fluid was used for a lamb or kid. This was varied a little with the experiment in progress, but previous work had shown that when conditions were suitable within the rumen the size of inoculum could vary within wide limits. Each inoculum was examined microscopically before use. Very occasionally an inoculum was given to adult animals via the cannula. Some limited populations were inititated by placing a few organisms in a buffered suspension directly into the mouth or cannula of the animal. The small numbers of organisms had been separated and counted under the dissection microscope. All other inocula used comprised many thousands of ciliates.

The vivar technique

The basic technique was first introduced by Fina *et al.* (1962) for use in cattle. The method involves the suspension in the rumen of small chambers or vivars which are fitted with Millipore membranes (Millipore (U.K.) Limited, Wembley, Middlesex)

which are permeable to rumen solutes but impermeable to rumen ciliates. In the present work rumen ciliate populations were maintained within vivars suspended in sheep.

The vivar apparatus. Plate 1, fig. 1, illustrate the components of the vivar chamber. Each consisted of an ebonite vessel of 10 ml. capacity at the base of which there was a Millipore membrane (*z*) of 25 mm. diameter held in place by the collar (*y*). The pore size of the membranes was 0·65 μ. A wire gauze (stainless steel) protected the membrane from the rumen contents of the host sheep and another slightly smaller gauze lay between the contents of the vivar and the membrane to prevent any damage to the membrane while sampling. The order from exterior inwards was, therefore: wire gauze, rubber washer, membrane, rubber washer, internal wire gauze. A slight indentation in the collar accommodated these and allowed the membrane to lie perfectly smoothly. The smaller wire gauze was cut to fit exactly into the base of part (*x*) of the apparatus and a small ridge prevented it from entering the vessel. As noted by Fina *et al.* (1962), provided the membrane was protected from mechanical damage it remained intact when immersed in rumen fluid.

Three of these vivar culture vessels could be suspended for as long as required in the rumen of a sheep fitted with a 2 inch cannula; Pl. 1, fig. 2 illustrates how this was done.

The stem of each vivar chamber was attached to heavy rubber tubing as illustrated by Pl. 1, fig. 2(*a*). The normal cannula cap was replaced by a cap (*b*) from which the centre had been removed. The three tubes by which the vivars were suspended were passed through holes in an ebonite disc (*c*) and this sealed the top of the cannula when cap (*b*) was screwed into place. The exterior end of each rubber tube was attached at (*d*) to one arm of a light aluminium or plastic T-piece and the other arm was sealed by a rubber cap (*e*). At times of sampling this cap was removed and a thin polythene tube, cut to a suitable length to reach the base of the vivar, was passed in. Withdrawals or additions were made with the aid of a 10 ml. glass syringe, and the contents of the vivar could first be gently mixed by several movements of the syringe. While this was in progress nitrogen from a balloon was allowed to flush the system through the stem (*f*) of the T-piece. Some time before sampling the syringe had been attached to the sampling tube and both had been filled with nitrogen and the tube sealed until the sample was to be taken. After sampling, the stem of the T-piece acted as a gas outlet by being connected as shown (*g*) to a narrow polythene tube, the other end of which was passed through the cap of an 8 ml. polythene centrifuge tube (*h*) and under the surface of a small volume of water held there. This prevented the entrance of atmospheric oxygen. Finally, all three tubes were tied with string until they were in the position shown by (*i*). Thus the extension of the apparatus from the sheep's cannula was decreased to a minimum, and the sheep could feed and move freely in its pen and did not appear to be affected by the apparatus. The plastic sheet (*j*) was placed over the cannula as shown to decrease contamination from any rumen fluid which leaked on to the sheep's wool round the edge of the cannula.

When suspending the vivars in the rumen it was essential for ease of sampling to make sure that they were spaced slightly apart. A length of fence wire covered with plastic was bent into a hook at the end and used to push each vivar into the rumen at the correct angle. Once they were placed in this way they very rarely became entangled.

Preparation of ciliate-free, centrifuged, rumen fluid. Rumen fluid obtained from the

ciliate-free cannulated sheep was centrifuged for 5 min. at 630 *g* and the supernatant fluid used.

Preparation of washed suspensions of protozoa. The previously described method with separating funnels was used (Eadie & Oxford, 1955) but with the bicarbonate buffer described by Abou Akkada & Howard (1960). Suspensions from smaller volumes of rumen fluid were obtained by decantation in 100 ml. boiling tubes.

Additions to vivar. Pure rice starch was obtained from Reckitt & Sons, Ltd., Hull. Two ground-nut meals (GN. 15, GN. 17) were used; these were chosen because of their high protein content and low nitrogen solubility in water (15% and 8% respectively, of the total nitrogen was soluble in water.) Ground-nut meal GN. 15 was known to have a 'very high' aflatoxin content (2·5 p.p.m. of aflatoxin). Acetone-dried barley-leaf protein as prepared by Duckworth & Woodham (1961) was also used. It had an even lower nitrogen solubility in water than had meals GN. 15 and GN. 17.

Routine procedure. The Millipore membranes were soaked in buffer before being fitted into the vivar chamber as described above. Then 5 ml. of ciliate-free centrifuged rumen fluid and 5 ml. of the chosen ciliate suspension were added by pipette to the chamber. The ciliate suspensions were thoroughly mixed and pipetted rapidly. Frequently a small amount of rice starch (10–15 mg.) was also added at this time, the amount used being gauged by the number and state of fill of the ciliates. While a gentle flow of nitrogen was being passed through the rubber tubing (Pl. 1, fig. 2(*a*)) the vivar was carefully fixed to the end. The whole assembly was then carried to the sheep, any subsequent changes to the contents being carried out *in situ* with the aid of a sampling tube and syringe as described.

The components of the suspension, and the number and degree of fill, of the organisms, could be altered as required, but the number of controls per experiment was necessarily limited since only six vivars could be run at one time (three per host sheep). Care was taken to mix the contents of the vivar immediately before withdrawal of samples in order to use comparable numbers of protozoa. The contents of the vivar were regularly sampled and rice starch suspensions added as required. It was necessary to judge from the appearance of the ciliates before feeding the amount of starch required, as this varied with the number of organisms and the time since the vivar had started. The barley-leaf protein and ground-nut meals were similarly added as suspensions.

RESULTS

IN VIVO EXPERIMENTS

Observations on the establishment of Ophryoscolex tricoronatus

This organism, although established, had subsequently died out in several sheep (Eadie, 1962*b*). However, the species was again established in three cannulated sheep at the Institute by inoculation from animals brought in from other areas and it was later established along with small *Entodinium* spp. in isolated sheep 661.

Since there had been some indication that Ophryoscolex was slow to establish when given to young lambs in a mixed inoculum Eadie (1962*a*), further inoculation experiments were made with both ciliate-free lambs and kids. In all cases the rumen conditions would be expected to be suited to ciliate development. The time taken to establish the organism when using a mixed type A inoculum including Ophryoscolex, was compared with that when Ophryoscolex was the only type A organism present (sheep 661).

It is clear from Table 2 that except in lamb 56 Ophryoscolex regularly failed to become established after the first few inoculations into a young animal. This was true whether Ophryoscolex was the only large ciliate in the population or was a component of a mixed population. Though the first inoculum was successful in lamb 56 this animal was possibly atypical since it was not well at the time of the first inoculation, and its ciliate population died while the animal was being treated for pneumonia. It required two further inoculations to re-establish the organisms. Other than in lamb 56 the population developed at a slightly earlier age in the kids than the lambs, as had been

Table 2. *The establishment of Ophryoscolex populations in sheep and goats*

Year	Kid (G) or lamb number	Type of inoculum	Age inocula begun (days)	No. of inocula used*	Age Ophryoscolex established (days)	Time taken from beginning of inocula (days)
1962	51	Mixed population including Ophryoscolex	26	5	61	35
1962	52		21	4	None by 50	over 29
1962	55		49	6	104	55
1963	77		23	5	93	70
1962	G111		18	5	51	33
1962	57	Ophryoscolex plus small entodinia	24	4	65	41
			26	1	33	7
1962	56		Re-inoculation	2	59	14
			45			
			57	1	65†	8
1964	17		Re-inoculation	1	90	6
			84			
1964	18		57	2	90	33
1962	G108		13	3	34	21
1962	53	Ophryoscolex plus Eudiplodinium	130	1	135	5
1964	87		122	1	137	15

* The interval between inocula was between 7 and 10 days except in 77, 17 and 18—see text.

† One active organism was seen at 65 days, but Ophryoscolex was not seen in subsequent samples until after re-inoculation.

suggested by previous observations (Eadie, 1962*a*). The regular inoculations were discontinued for intervals of 2 weeks and 4 weeks in lamb 77, but Ophryoscolex did not develop. In lambs 17 and 18 a month interval was left after the first inocula, but second inocula were necessary. These observations proved that the Ophryoscolex was not already established and merely developing more slowly after the first inoculum than components of other limited populations. As seen in lambs 53 and 87, the time required after inoculation and the number of inocula necessary tended to be less at a greater age, and in the isolated animal 661 a population was established at 11 months of age after one inoculation with not more than 27 organisms. Nevertheless, Ophryoscolex was still observed to die out in older animals and could not always be re-established (see later). Shortened, 'stumpy', caudal processes were frequently noted in the Ophyroscolex when a population was first becoming established.

Observations on the development of other species in young animals

Type A organisms other than Ophryoscolex have been shown to establish very rapidly in young animals. When rumen conditions were suitable for ciliates, a first inoculum at 3 weeks of age usually resulted in establishment (Eadie, 1962*a*).

When inocula of mixed ciliates of type B from cattle were given to two lambs 78 and 79 at 5 weeks of age, all but the tailed species of Epidinium were readily established. *Epidinium ecaudatum* developed 1 week after the first inoculation, but the tailed species of Epidinium were only seen at 4 and 5 weeks after second inoculations. It was later noted that Ostracodinium, a genus more typical of cattle than sheep, had died out of both lambs—by 6 months after the first inoculum in 78, and by 9 months after the first inoculum in lamb 79. In a third lamb given a mixed type B population as inoculum all Epidinium species failed to establish. *Dasytricha ruminantium* is the only other species in which variation in establishment time has been noted.

Establishment of limited populations containing Ophryoscolex and one other large Ophryoscolecid

Ophryoscolex plus Polyplastron. It proved quite easy to establish and maintain a population containing these organisms and small entodinia in both a kid (G 108) and a lamb (81). (see Table 3). This limited population in kid G 108 was of value in vivar experiments on antagonism (see later).

Table 3. *Establishment of apparently stable limited ciliate populations* in young animals which were initially ciliate-free*

Kid (G) or lamb number	Sequence	and components of inocula	Resultant stable populations
53	1	Eudiplodinium + Ophryoscolex	Eudiplodinium + Ophryoscolex
87	1	Eudiplodinium + Ophryoscolex	Eudiplodinium + Ophryoscolex
	2	Epidinium	Epidinium + Eudiplodinium
57	1	Ophryoscolex	Ophryoscolex
	2	Eudiplodinium	Eudiplodinium + Ophryoscolex
	3	Polyplastron	Polyplastron + Ophryoscolex
81	1	Ophryoscolex + Polyplastron	Ophryoscolex + Polyplastron
G 108	1	Ophryoscolex	Ophryoscolex
	2	Eudiplodinium	Eudiplodinium + Ophryoscolex
	3	Polyplastron	Polyplastron + Ophryoscolex
G 110	1	Eudiplodinium	Eudiplodinium
	2	Polyplastron	Polyplastron
	3	Eudiplodinium	Polyplastron
G 200	1	Eudiplodinium	Eudiplodinium
	2	Polyplastron	Polyplastron
G 202	1	Polyplastron	Polyplastron

* In every case small entodinia were also present.

Ophryoscolex plus Eudiplodinium. It was of interest to determine whether Ophryoscolex, frequently found in a type A population, and Eudiplodinium, an important component of the type B population, would develop as the only large Ophryoscolecids in a rumen population. This proved possible in sheep 53 and 87 using a mixture of the two protozoa from sheep 661 and 95 as inoculum. The mixed population remained in 53 for $3\frac{1}{2}$ years but at this point Ophryoscolex dwindled in number and disappeared—a change similar to those noted below in mixed type A populations. The same limited population was successfully established by inoculation of the separate species into lamb 57 and into kid G 108 (see Table 3).

Ophryoscolex plus Epidinium. It was not possible to establish these two genera in the same animal, whether alone or in a mixed population; this problem is being further investigated. As an example, when Epidinium was inoculated into sheep 87 following the establishment of Ophryoscolex and Eudiplodinium (above) the Ophryoscolex gradually disappeared and Epidinium developed along with Eudiplodinium. So far Epidinium consistently appears to develop in preference to Ophryoscolex when the two organisms are mixed *in vivo*.

The disappearance of Ophryoscolex from mixed type A populations. The three cannulated sheep used to establish Ophryoscolex in an already established type A population were $1\frac{1}{2}$, $2\frac{1}{2}$ and 10 years old. The oldest animal was one from which Ophryoscolex had previously disappeared (Eadie, 1962*b*), yet the organism re-developed and was present in fair numbers when the sheep was killed 4 months after the inoculation. A second animal was killed $1\frac{1}{2}$ years after inoculation; Ophryoscolex was still found to be present. In the third and youngest animal the Ophryoscolex dwindled in numbers and were not seen after 17 months from the inoculation. These observations would indicate that the disappearance of Ophryoscolex from a population is not directly related to the age of the animal.

The instability of Ophyroscolex in ciliate populations was further examined by using sheep 146. This animal had a mixed type A population which had previously included Ophryoscolex, but though regularly inoculated with Ophryoscolex from the isolated sheep 661 the species was not re-established. To investigate this further the following two experiments were carried out.

(i) Lamb 81 with a well-established population of Polyplastron and Ophryoscolex was inoculated with rumen material from sheep 146. The other ciliates from sheep 146 developed in lamb 81 yet Ophryoscolex remained numerous for 10 weeks. When numbers of Ophryoscolex later decreased the population was increased again by inoculation. It seemed that there might be some relationship between Diplopiastron and Ophryoscolex, but there appeared to be no direct or rapid effect of the sheep 146 rumen inoculum *per se*, yet over the same period of time inocula from sheep 661 did not re-establish Ophryoscolex in sheep 146.

(ii) This experiment was done with twin lambs 17 and 18, in which Ophryoscolex had been established as the single large species by inoculation (Table 2) and had developed well. At 125 days of age lamb 17 was inoculated with a mixed type A population from sheep 208 in which Ophryoscolex had been established for several years and lamb 18 was inoculated from sheep 146 in which Ophryoscolex had repeatedly failed to establish. In other respects the ciliate populations were similar. In this experiment it was lamb 17 which showed a great decrease in Ophryoscolex but, though fluctuating in number, the organisms remained. At 223 days of age, when the experiment was terminated, Ophyroscolex was still present in both lambs. Once again there was no indication that the 146 population in itself could remove Ophryoscolex from another animal.

Further in vivo investigation of the antagonism between type A and type B populations

Inoculation experiments were made partly to confirm and extend previous findings and partly to make comparisons with the vivar experiments (see later). Type A populations had become dominant in both sheep and cattle rumens but a type B population

was seen to become dominant in calves F, G and 35 (Eadie, 1962*b*). Large Eudiplodinium organisms, never observed in sheep, were associated with this latter change and also with extended periods of time in which organisms of both types were together in cattle. Thus the presence of large eudiplodinia might be significant in the reversal of the antagonism. The previous findings suggested that the type A organism Polyplastron and the type B organism Eudiplodinium were the only mutually antagonistic species, but studies of the relationship between Epidinium and Ophryoscolex may cause some revision of this view. Ophryoscolex was not present in the type A populations used in 1962, (Eadie, 1962*b*), hence the present interest in this species. Similarly changes of population in goats had not been examined.

Cattle. Calf Z, which had been used as an isolated animal (Eadie, 1962*a*), was inoculated with a mixed type B population from a cow. When this population had been established a type A population was inoculated and, after 3 weeks in which both were present, type A became dominant. When re-inoculated with a type B population calf Z showed an effect similar to that previously observed in cattle (see above); that is, an extended period in which both Polyplastron and Eudiplodinium were seen in fair numbers and in which Eudiplodinium of an unusually large size were present amongst the re-established population. The other type B organisms did not become re-established. After 2 months with the combined (A + B) population type A again became dominant. Ophyroscolex was included in the type A population but only very small numbers were ever seen.

Goats. In three animals G108, G110 and G200 the dominance of Polyplastron (type A), when inoculated from the isolated sheep 683, was demonstrated (see Table 3). In all three Eudiplodinium was the only type B organism initially present but in goat G108 Ophryoscolex was also present initially. The Ophryoscolex remained along with Polyplastron after the change. The time taken to complete the change in population was 9, 11 and 12 days. This contrasts with the longer time taken in some calves (e.g., calf Z above). In two of the goats unusually large Eudiplodinium organisms similar to those noted in calf Z were seen during the period when a combined type (A + B) population was present (see Pl. 2, figs. 1, 2). The Polyplastron also tended to be large and were often microscopically extremely dense just before the change-over—a feature of the antagonism frequently noted (Pl. 2, fig. 3). After the change some Polyplastron remained large for some time and were used in vivars (see later). Neither large- nor normal-sized eudiplodinia were ever observed again following inoculations of large numbers of eudiplodinia to goat G110.

Thus the population in goats displayed features of the antagonism intermediary between sheep and cattle. As in sheep, Eudiplodinium did not become re-established even temporarily but large Eudiplodinium were observed, at the time of the change. In other respects, for example, the numbers of large bacteria such as Oscillospira which develop in the young ciliate-free animal, kids seemed similar to lambs (Eadie, 1962*b*).

Sheep. As was expected, when Polyplastron and small entodinia from the isolated sheep 683 were inoculated into lamb 57, which had a population of Eudiplodinium and Ophryoscolex, the Polyplastron became dominant and all Eudiplodinium disappeared (Table 3); as in the goat G108 the Ophryoscolex also remained but no large Eudiplodinium were observed. This confirmed previous observations. As the previous experiments indicated, very dense, frequently dividing, and quite large

Polyplastron were associated with a population change (Pl. 2, fig. 4; see Eadie, 1962*b*, for lamb 222). Organisms in which internal structures such as the two large skeletal plates were clearly visible (see Pl. 2, fig. 6*b*) were more typical of Polyplastron from animals on the diets used here.

In an effort to induce the development of large Eudiplodinium organisms in a sheep, two inocula of 100 ml. and 200 ml. of whole rumen fluid from calf Z, when a combined type (A+B) population was present and large eudiplodinia were conspicuous, were given to a sheep. Before the inoculation only Isotricha, Polyplastron and Entodinium were present in the sheep. The inoculations led to the development of Diploplastron, Dasytricha, and even a few Ophryoscolex which had been very infrequent in calf Z. However, no Eudiplodinium either large or small were observed. Similarly, a smaller inoculum into another sheep with a type A population failed to establish the large Eudiplodinium, even temporarily, though Diploplastron of type A was added to the population.

With one exception, experiments to date have confirmed the previous findings that a type A population would readily and irreversibly remove type B organisms from a sheep rumen fauna; the exception was with sheep 209. Though this animal was placed between, and in contact with, two sheep with mixed type A populations for over 9 months, its type B population was retained. Even after one small inoculum (about 40 organisms) and two larger inocula of Polyplastron plus small entodinia (concentrated from 175 ml. and 600 ml. sheep 71 rumen fluid) the Eudiplodinium of type B population remained. Though Polyplastron were seen for a short time large Eudiplodinium were never observed. Three weeks after Polyplastron was first seen Epidinium disappeared. Two other sheep which had earlier received a type B population direct from sheep 209 showed a typical change of population, in one case due to accidental contamination with what must have been very few organisms. It appeared that sheep 209 might have shown an animal difference but unfortunately the animal died of pneumonia before further experiments could be completed; at the time of death Polyplastron and Eudiplodinium were both present in the population. The rumen fluid had on occasions been as low as pH 5·5, and the postmortem examination indicated that the animal had been suffering for some time from subclinical pneumonia. It is possible that these factors may have affected the population.

VIVAR EXPERIMENTS

It was clear from the present study and previous work that experiments *in vivo* were not suitable to determine the cause of the antagonism between type A and type B populations, but could only demonstrate under what conditions it existed. The chief object of vivar experiments was to determine the cause. The vivars had the great advantage that both populations could be examined together in a closed system, unlike the case in sheep *in vivo*, for example, where re-inoculated type B organisms have never been seen.

Maintenance of normal ciliates within a vivar

Preliminary experiments were made with different concentrations of ciliates in the initial inoculum and variations in the frequency and amounts of rice starch fed. The numbers of ciliates which could be used varied over a wide range, and naturally

varied with the components of the population: later counts of fixed inoculum suspensions showed that in general, in a satisfactory run, the concentration in the vivar at the onset of the experiment had been 2–5000 large Ophryoscolecids/ml. The organisms were usually fed with rice starch twice daily, the amount ranging from 10 to 25 mg., depending on the numbers and species present and the length of time that the vivar had been running. The rice starch was always added as a suspension in buffer, the volume usually being the same as the last vivar sample taken. The initial rate of starch utilization was noticeably greater than at a later stage and the gauging of the first overnight feeding was particularly important. However, with experience, organisms could be maintained in an apparently normal active state for 4–6 days. Beyond that time, it seemed inadvisable to rely on the results because the ciliates, though still alive, appeared to be less healthy, and the background bacteria seemed to become more prominent.

The Millipore membrane completely prevented the passage of ciliates in either direction, and since the centrifuged rumen fluid was taken from a ciliate-free animal there was no possibility of ciliates being added in that way. The following populations could be maintained for several days: a mixed type A population, a mixed type B population, Eudiplodinium plus Ophryoscolex, Polyplastron plus Ophryoscolex, also Polyplastron, Eudiplodinium and Ophryoscolex alone save for small entodinia. It was noticeable that the proportions of certain species within a mixed population in a vivar tended to change with time. Thus there seemed to be some competition between large entodinia (*Entodinium bursa* Stein) and *Diploplastron affine* in the mixed type A population though there was no sign of one population rapidly eliminating the other. Similarly in a mixture of the two, the numbers of Ophryoscolex were not maintained as well as Eudiplodinium, but again division rate and competition for rice starch under the restricted conditions of the vivar might account for this. When only Ophryoscolex and entodinia had been inoculated into a vivar the proportion of Ophryoscolex with short 'stumpy' caudal processes rose, even after only 12 hr. (e.g. from less than 10% in the inoculum to an average of over 60% in the three vivars of one run). This might be compared with the occurrence of these forms *in vivo* in a young animal or isolated animal when Ophryoscolex was first becoming established.

Though it was possible to maintain Ophryoscolecid ciliates which ingested rice starch, the vivar technique was not so satisfactory for the holotrich organisms, especially Dasytricha, which tended to become abnormally empty within a few days. It seemed that the diffusible sugars from the host sheep did not become available in sufficient quantity; though clearly some nutrient was available or Dasytricha which does not ingest starch granules would rapidly have died.

Bacterial development within a vivar

The Millipore membrane would exclude all but the smallest bacteria. On a number of occasions, Gram-stained films of the vivar contents were examined. These indicated that for any one controlled run (that is three vivars in one sheep) the bacterial picture was similar in each vivar. During an experiment an increase in small Gram-positive rods was observed, especially when a ciliate population had been allowed to die within the vivar, but there was no strikingly abnormal development of bacteria. A control vivar without ciliates did not show the same increase in small Gram-positive rods until starch was added, showing that the starch within the dead ciliates had possibly

stimulated the rod development. On the whole, therefore, it could be assumed that over the first few days bacterial conditions in the vivar were not unlike those within the rumen, though many of the large bacteria had been removed by the light centrifugation of the rumen fluid added to the vivar. The bacterial picture was dependent on that of the centrifuged rumen fluid used initially and small amounts of this were always kept for further examination in case there was any unusual development within the vivar.

The effect of host sheep with different types of population

It was initially believed that a diffusible material similar to the toxic biotic substances which affect certain algae (Rice, 1954; Proctor, 1957) might be the cause of the observed antagonism between type A and type B populations. Thus the viability of a type B population within a vivar when suspended in a sheep with a type A population was compared with that when a vivar was suspended in a host sheep with a type B population. The ciliates within the vivar appeared to be unaffected by the host sheep population. Since the Millipore membrane would allow the passage of a diffusible material it was assumed that antagonism between type A and B populations must be due to a more direct effect.

The effect of mixing populations of type A and type B within a vivar

When populations of type A and type B were mixed within a vivar the antagonism observed *in vivo* in sheep was reproduced, i.e. dominance of type A Polyplastron. The type B organisms had generally disappeared by 1–2 days after the two populations were mixed, and it was immaterial which population was placed in the vivar first. Even 12–24 hr establishment of one population did not alter the speed of the change. When Polyplastron was about to become dominant the organisms were often observed to be very similar to those seen *in vivo* at the time of a population change, very densely filled organisms, sometimes quite large, with division frequently seen (see Pl. 2, figs. 3, 4, 5).

Procedure used in further experiments

To make control and repeat experiments a standard procedure had to be used. Because of the problems of food competition and the greater difficulty of assessing food requirements in a mixed population it proved more convenient to use a limited population in most experiments. Changes using mixed type A populations were made but unless the change was effected rapidly it was not easy to assess the effect produced by the rather large numbers of background ciliates. The presence of a few small entodinia in the washed suspensions used from limited populations proved a great advantage, since they indicated, by their state of fill, the excess starch which had been available. With practice it became easy to assess and adjust the concentration of a population to be used for an inoculum; suitable suspensions could be chosen by examination of comparable volumes under the microscope. Where controls from different suspensions were being used a rough count was made on several drops of equal volume. As speed was vital these assessments had to be made more rapidly than would be possible with an accurate counting technique. However, in any one experiment there appeared to be good repetition and slight variations in subsequent runs were a useful check of the reproducibility of an observation. Usually, the volume of fluid removed at

sampling was replaced by the buffer of the food suspension. As the capacity of a vivar was small, it was not possible to do any accurate counts on the population during a run, and because of unknown changes of volume through diffusion such counts would be of little value. However, since the ciliates could not escape from the vivar, changes in relative proportions could easily be recorded. The state of fill of the organisms could also be judged quite easily and this was of experimental interest as well as being necessary in the assessment of food requirements. In general each vivar run was started at mid-day and any additional ciliate organisms were added 12–18 hr later, when the first population had become established. When the population change followed 1–2 days later, the organisms were generally left to return to 'normal', i.e. a size and microscopic density similar to that normally found *in vivo*. Thus an average run lasted about 5 days, which was within the time limit of the technique. The contents of each vivar were examined from 1 to 3 times/day and at least three vivars were used for each run.

Investigation of the nature of the antagonism

Since the antagonism occurred when the two populations were in direct contact it seemed that the cause might be either food competition, as shown between ciliate genera in activated sludge by Brown (1964), or predation. As mentioned above, during a population change the polyplastrons became so dense that it was impossible to distinguish the contents under the light microscope (as *in vivo* experiments). These organisms returned to 'normal' 1–2 days later. In contrast, when cannibalism was occasionally noted in Polyplastron from sheep which had been without food overnight (12 hr) the ingested organisms could be seen quite easily (Pl. 2, fig. 6). In any case the observation of what Lubinsky (1957) called 'accidental predation' would not be proof that predation was the cause of the antagonism. Ingestion could be seen very occasionally in a natural ciliate population, for example, holotrichs and entodinia within Polyplastron, yet these ingested species were not eliminated from a population. Similarly Lubinsky (1957) recorded Ophryoscolex within Polyplastron, although he also pointed out that Dogiel (1947), who must have studied many thousands of rumen ciliates, stated that predation did not occur in the larger Ophyroscolecids. Thus to prove that selective predation was the cause of the population changes it was essential to eliminate the superficially more probably cause, namely food competition.

Initial experimental evidence against food competition

In several experiments, at the time type B organisms had been eliminated from a mixture of type A and type B populations, the type B organisms living alone in a control vivar were seen to be well filled and active. This was observed even where the quantities of rice starch had been adjusted so that considerably less was given to the control than to the vivars containing both types of organism.

After a change of population the small entodinia were often well filled. This would indicate that the starch had not been completely used up by the type A organisms and that some should therefore have been available for type B organisms as well as entodinia.

The occasional appearance of ciliate fragments during a change-over indicated some deaths within the vivar. However, if the elimination of type B organisms was entirely due to death by starvation during a vivar run, then many fragments of the organisms should have been visible; this was not the case. If food competition had

occurred the only other explanations were that these fragments were ingested by Polyplastron or had disintegrated rapidly. An experiment was therefore made to to examine these possibilities. Active polyplastron were placed in a vivar containing a suspension of type B organisms previously allowed to die by starvation in a vivar. There seemed to be no decrease in the number of fragments where Polyplastron was present, as compared with a control without. Also the fragments remained clearly distinguishable over 5 days.

Thus it seemed clear that competition for food, in the form of starch, was not the primary cause of the disappearance of the type B organisms. The other suggested cause, predation, was confirmed by the observations below.

Direct observation of predation and cannibalism within a vivar

During repeated examinations of vivar contents at the time of a change-over of population it was possible, very occasionally, to see ingested organisms; on one occasion numerous polyplastrons were seen to have ingested type B organisms. Although the actual process of ingestion was not observed, one ingested organism was seen alive for several minutes. However, within 20 min. the polyplastrons began to develop the typical extremely dense appearance, after which the contents could not be distinguished. This rapid change explains the difficulty of determining the cause *in vivo*.

The fact that the mouthparts of Polyplastron are extremely elastic and strong was illustrated by a form of cannibalism in Polyplastron which occasionally followed a population change in a vivar. As illustrated in Pl. 2, fig. 7, though Polyplastron is fairly rigid and is not easily distorted mechanically, it was considerably misshapen. A few polyplastrons were seen with extended mouthparts which were attached to other polyplastrons as if by suckers. This particular form of cannibalism has not been seen *in vivo*; it was possibly a result of the confined conditions within the vivar. Gelei (1925) noted a similar chewing effect in cultures of cannibalistic Stentor.

Having determined the most probable mechanism of the antagonism between Polyplastron and type B organisms further experiments were made to investigate the factors involved. In all experiments, though predation was known to be the chief cause of the antagonism, it was essential to beware of masking the result of a run by an increase of food-competition effects. Unless an effect was exceptionally striking it was necessary to do a number of controlled repeat experiments before drawing the conclusions cited below.

Observations on factors affecting the antagonism between certain protozoa, based on vivar experiments. The observations may be recorded in the following subsections.

General observations on predation in Polyplastron

1. It was immaterial which population type was established first in a vivar or the two could be mixed *in vitro* immediately before inoculating the vivar. Thus there seemed to be no question of 'conditioning' the vivar contents. Provided the same sources of ciliate populations were used the change in population (that is, the disappearance of type B organisms) occurred after the same time interval, usually between 24 and 48 hr.

2. As in sheep, the smaller type B organisms were removed first and Epidinium before Eudiplodinium. The latter was always the last to be removed.

3. As in the *in vivo* work (Eadie, 1962*b*), changes of population from type B to type A in vivars were often followed by a decrease in the numbers of Polyplastron.

Cannibalism and its comparison with predation

4. Cannibalism was observed under two conditions. (*a*) When Polyplastron of a limited type A population had been left overnight in a vivar and had become moderately empty. This form of cannibalism was similar to that occasionally seen *in vivo* when the protozoa had been some time without food (Pl. 2, fig. 6) and would agree with Dawson's (1919) observation that cannibalism occurred when food was *beginning* to be depleted. (*b*) On occasion, immediately after the removal of a type B population or when very few type B organisms still survived, a few chewed misshapen polyplastrons were conspicuous amongst the well-filled organisms which remained (Pl. 2, fig. 7).

5. An experiment in which moderately filled Polyplastron were fed on mixed type B organisms and the control was left without food showed that, whereas the predators lived for 1–2 days, the polyplastrons alone, though showing signs of cannibalism, died in a manner typical of starvation. Thus cannibalism was less effective nutritionally than predation.

Ciliates not removed by Polyplastron

6. Small entodinia were not removed even when they were the only organisms along with active cannibal polyplastrons.

7. Observations made on Ophryoscolex in sheep and goats were confirmed, i.e. Ophryoscolex would remain, when present in the original mixture, regardless of whether it was initially from a population with Eudiplodinium (type B) or Polyplastron (type A). Ophyroscolex was decreased in numbers, but this possibly was due to food competition or slower division rate and occurred in a vivar when Ophryoscolex was maintained with species other than Polyplastron (see p. 185).

8. When the mixed type A population from sheep 146 was used in vivars it removed a mixed type B cattle population, and also Eudiplodinium from a mixture of Eudiplodinium plus Ophryoscolex. Thus for the duration of a run, Ophyroscolex remained in a vivar along with the sheep 146 population, and this at a time when large inocula of Ophryoscolex placed directly into sheep 146 via the cannula did not establish the organism (see p. 182).

Conditions under which Polyplastron did not remove type B organisms

9. Starvation, rather than stimulating predation, appeared to prevent it. Moderately full, apparently healthy polyplastrons were necessary. An experiment to compare moderately full organisms immediately after removal from a sheep and nutritionally similar ones from the same population but kept for several days in a vivar, indicated similar predatory activities. However, when the predatory activity of Polyplastron starved in a vivar was compared with moderately full organisms from a sheep, the starved organisms did not show any sign of removing a type B population. Also, there was some indication that very empty polyplastrons, on the point of death, became unusually non-selective and would ingest fragments of dead ciliates, thus contrasting with normal or predatory polyplastrons which did not ingest dead remains (see p. 188).

10. Other than starvation and the experiments with sheep 209 (see below), the only observed cause of failure of a change-over was the absence of a minimum number of

polyplastrons. This was shown by inoculating into a vivar containing a type B population a small number of polyplastrons (about 30), and comparing this with the effect of the usual inoculum of some thousands. The small number of polyplastrons lived and were clearly predatory, but did not effect the change within the 4 days of experiment. However, there was no sign of a reversal to type B only, nor were any large Eudiplodinium observed. The minimum number of polyplastrons required could not be stated categorically because it depended to a certain extent on the predatory activity of the particular type A population used.

Variations in the predatory activity of Polyplastron

11. There was some indication of variation in predatory activity of Polyplastron from causes other than starvation. Predatory activity could be judged by comparing the speeds of removal of a given type B population, or the speed of apparent increase in numbers of Polyplastron relative to the Eudiplodinium, should complete removal not occur. As mentioned (p. 183) there was a tendency for larger polyplastrons to be present during a change-over, *in vivo* or in a vivar. In goats G108 and G110 larger polyplastrons remained in the animal for 1–2 weeks after the change-over and in several vivar experiments these organisms showed more rapid predation than control organisms from sheep 683.

12. There was some evidence of a similar increase in predatory activity when polyplastrons which had already removed a type B population in a vivar were compared with polyplastrons direct from a sheep. This increase in activity was less convincing than that mentioned in section 11 above, but in several experiments there appeared to be a more rapid increase in the proportion of Polyplastron relative to the type B organisms. Also, when polyplastrons given a second inoculum of type B organisms were compared with polyplastrons which had been maintained alone in the vivar, those which had previously removed a type B population were more active predators.

13. When twelve large-sized polyplastrons from a double 'take-over' (section 12 above) were inoculated into a ciliate-free lamb, small polyplastrons developed. These later died out, but the significance of this is not known.

14. Large size is often associated with predation and cannibalism in ciliates (Giese, 1938; Giese & Alden, 1938; Dawson, 1919). Some organisms, for example, Blepharisma, Stylonychia and Oxytricha, appear to get progressively larger with an increase in size of prey (Giese, 1938; Padmavathi, 1961). This was not the case with Polyplastron. When polyplastrons in a vivar were fed small Eremoplastron, followed by larger type B organisms, the polyplastrons did not become progressively larger, and as in other experiments they became normal (that is small again and less dense) 1–1½ days after cessation of feeding.

15. Unusually large polyplastrons from the barley-fed sheep 300 were considerably more active predators than average-sized control polyplastrons. The change-over with the former could occur within 12 hr, but with the latter took 1–2 days (see section 1 above).

Predatory activity in the presence of an insoluble protein

16. Both ground-nut meal (meals GN. 15 and GN. 17) and barley-leaf protein were used to see whether they acted as a sparer of type B organisms. These two protein materials were very insoluble in water so that they remained particulate within the

vivar. Both materials were readily ingested by the ciliates, and though ground-nut meal GN. 15 had a high aflatoxin content there was no sign of toxicity for the protozoa. There was an indication that predation was slightly slowed down when the proteins were available, but it was not prevented. There was a very clear indication that the presence of protein increased the rate of utilization of rice starch during a population change, since organisms with available protein became less well filled than the controls and had to be fed with more rice starch than the latter. This was also true whether Eudiplodinium, Ophyroscolex or Polyplastron was the only large Ophryoscolecid in a vivar.

Attempts to initiate dominance by type B organisms including experiments with sheep 209 population

17. Attempts were made to induce a change of population from type A to type B organisms by the alteration of conditions (such as relative numbers and state of fill) in the limited populations generally used. These attempts were unsuccessful and no large Eudiplodinium were ever seen (see p. 184).

18. The vivar technique had not been perfected when large Eudiplodinium were present in calf Z. Thus the nearest approach to a dominant type B population was that in sheep 209 when inocula from sheep 71 did not cause a population change from type B to type A. (p. 184). Vivar experiments were made with material from sheep 209 and 71, but the very large number of medium entodinia (e.g. *E. caudatum* and *E. longinucleatum*) in vivar inocula from sheep 209 made repeat experiments to assess the food requirements of the organisms essential, but unfortunately the sheep died before enough experiments were completed. However, the following experiments were made. (*a*) In one vivar run of the three completed with sheep 209 type B organisms and type A organisms from sheep 71, Polyplastron became dominant; that is, all type B organisms disappeared. In the other two runs the two populations lived together over the 5 days of the vivar run. The significance of the last two runs is difficult to assess because of the possible inadequacy of the food supply available to the Polyplastron due to competition from entodinia (see above). However, there was never any sign of type B organisms becoming large or dominant. (*b*) Since sheep 209 was a host it was possible to carry out a vivar run in that animal and to compare it with a control run in sheep 208. Again sheep 71 provided the type A population and in this case centrifuged rumen fluid from sheep 209 was used in the vivars in sheep 209. The object was to determine whether the factor preventing Polyplastron from sheep 71 from becoming dominant in the rumen population of sheep 209 would have any effect on the vivars suspended in that animal. This experiment took place just before the death of sheep 209 when both types of organisms were seen in that animal. Type B organisms were not removed from either set of vivars, large Eudiplodinium did not develop, and over the 5 days of the vivar run there was no indication that the polyplastrons suspended in sheep 209 were any less active than those in sheep 208. Thus, although type A organisms did not become dominant in sheep 209, a change of population did take place in one vivar, so that there seemed to be no direct effect of the sheep 209 population. The slowness of change in the other vivar experiments may have been a reflection of the state in sheep 209 *in vivo*, but it may equally have been due to a decrease in the predatory activity of Polyplastron through food shortage. The polyplastrons from sheep 71 were active in other experiments made concurrently.

The effect of mixing Ophryoscolex and Epidinium

19. Vivars were used to examine the relationship between Ophryoscolex and Epidinium, but over the period of a vivar run there was no indication of antagonism. A decrease in numbers of Ophryoscolex relative to Epidinium was noted but this was similar to that observed when Ophryoscolex was in a population with Eudiplodinium or Polyplastron.

DISCUSSION

The reason why certain rumen ciliates such as Ophryoscolex and Epidinium are less readily established in young lambs and kids than other ciliate species is not known, nor is it clear why Ophryoscolex disappears from populations in older animals. This latter change does not appear to be caused by the direct action of Polyplastron since Ophryoscolex is regularly a component of type A populations and this is confirmed by the observation that Ophryoscolex will also disappear from a population with the type B organism Eudiplodinium as the only other large Ophryoscolecid. It seems that this variation in the stability and ease of establishment of Ophryoscolex in older animals (see p. 182) may be a demonstration of differences between individual hosts (cf. Eadie, 1962*a*). It is possible that the failure of Polyplastron to become dominant just before the death of sheep 209 may be a further example of features of the host or its bacterial flora affecting the ciliate population rather than unusual features of the ciliate population *per se* being the cause of an apparent anomaly.

The disappearance of Ophryoscolex from a population which includes Epidinium differs from the above noted instability of Ophryoscolex in that it is directly related to the presence of Epidinium. However, failure to reproduce the antagonism in a vivar indicates that the speed of the effect is not as great as when Polyplastron becomes dominant by predation. The cause of the antagonism is not yet clear but it seems that other factors such as food competition, rather than predation, may be involved.

The observed reduction in size of caudal processes in a developing Ophryoscolex population shows that these organelles are not a reliable taxonomic feature. A reduction in the caudal processes of *Entodinium caudatum* has been noted by Coleman (1963) in cultures of that species and a similar, apparently permanent, reduction has been seen in *E. caudatum* maintained as the major ciliate species in an isolated sheep. Several years after the species had been established caudal processes were very rarely seen and did not redevelop when the population was transferred by inoculation to a young ciliate-free lamb.

Predation by Polyplastron has been shown to differ from the 'accidental' predation previously noted by Lubinsky (1957) in that it leads to the complete removal from the population of those species which form the prey. The reasons why some species should be eliminated while others remain, although occasionally ingested, are not known. The regular appearance of predatory or cannibalistic Polyplastron in sheep ciliate populations suggests that these features are common to all Polyplastron and are not a limited racial characteristic. This is also indicated by the apparent return of large organisms to a smaller size. The stimulus to predation in Polyplastron is still not clear, but as in the case of cannibalism in Stentor (Tartar, 1961) it is due neither to over-feeding nor starvation, nor is it merely caused by a lack of protein. Predation is more effective than cannibalism in keeping the protozoa alive. The ingestion of ground-nut meal and barley leaf protein, though not preventing predation within a vivar, leads to an in-

creased rate of utilization of carbohydrate. On the other hand predation does not markedly increase the carbohydrate utilization, which suggests that the ingested organisms are a less satisfactory protein source than the other proteins tested. Predatory 'activity' appears to be increased by previous predation. The significance to the organisms of predation is difficult to assess. As noted previously (Eadie, 1962*b*) the numbers of organisms tend to drop after a 'take-over' in sheep and this reduction in numbers could be due to a slower division rate or to cannibalism. There is no reason to believe that cannibalism is widespread amongst Polyplastron *in vivo* but it does occur at the onset of food shortage and under those conditions would have the effect of reducing the food requirements of the population, and perhaps ensuring survival of the species. A similar explanation does not, however, explain predation by Polyplastron. While predation of an original type B population leaves Polyplastron with more food and space per organism, there is no reason to believe that food supply was in fact limiting before predation began. Under the same conditions other type B organisms would have been successfully established in the population.

The complete and rapid elimination of a species from a particular habitat because of direct interaction with another species does not appear to be very common in nature (Elton, 1958; Andrewartha, 1961). The present observations are probably most nearly comparable with the observed relationship between the larvae of two species of blowflies, *Lucilia sericata* and *Chrysomyia albiceps*, cultured on meat (Ullyett, 1950). Though both species were living on the same food, of which there was an ample supply, *C. albiceps* first killed the larvae of Lucilia in preference to ingesting the food and thus Lucilia was eliminated.

Arising from the present work the most obvious question still to be answered is what in the course of evolution has saved type B organisms from extinction. There must be numerous transfers of ciliates by mouth to mouth contact between host animals yet type B and type A populations are both common. Clearly conditions under which Polyplastron fail to become dominant must occur quite frequently but the nature of these conditions has not yet been determined. In cattle and in goats large-sized Eudiplodinium have been observed and these appear to be associated with a tendency for a change from type A to type B in cattle (see p. 183). Large-sized Eudiplodinia have not been produced experimentally in a vivar with sheep as 'host' but work with cattle as 'host' is planned. The question of large size and its relationship to food and predation is being further investigated.

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. The components of a vivar chamber—for explanation see text.

Fig. 2. The method by which the vivars are suspended in the rumen—for explanation see text.

PLATE 2

All the organisms were fixed with formalin and were unstained.

Fig. 1–fig. 7 magnified × 105

Fig. 1. *Eudiplodinium maggii* from goat G 108 prior to the addition of *Polyplastron multivesiculatum* to the population.

Fig. 2. Unusually large Eudiplodinium from goat G 108 after the addition of Polyplastron and 5 days before the change-over of population. Ophryoscolex is also seen.

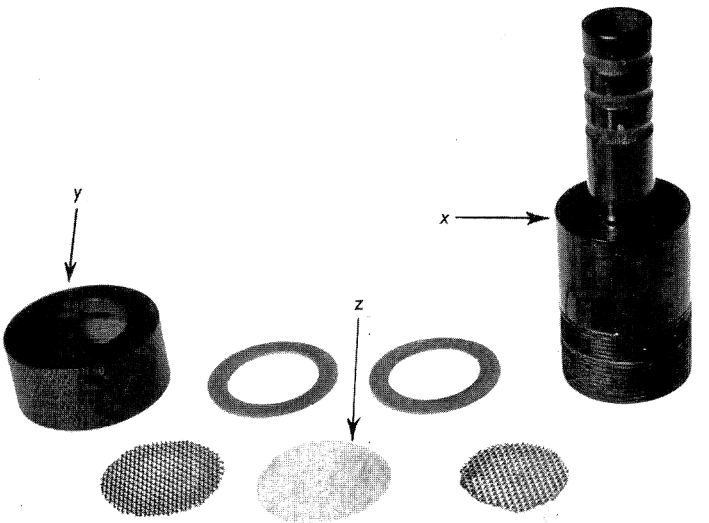
Fig. 3. Large, densely filled dividing, Polyplastron from the same sample as Fig. 2. Ophryoscolex and a 'normal'-sized Eudiplodinium are also seen.

Fig. 4. Densely filled Polyplastron from lamb 222 at the time of a change-over of population The type B organism Epidinium is also seen and a number of entodinia.

Fig. 5. Sample from a vivar at the time of a change-over of population. Densely filled Polyplastron from G 108 and a 'normal'-sized Eudiplodinium from sheep 95 are seen.

Fig. 6. Polyplastron from 683 before a.m. feed. Cannibalism is seen in organism *a*. Internal structure, e.g. the two large skeletal plates, can be seen in *b* and *c*.

Fig. 7. Sample from a vivar taken just after a change-over of population. Polyplastron which have become misshapen through 'chewing' by other Polyplastron are seen.



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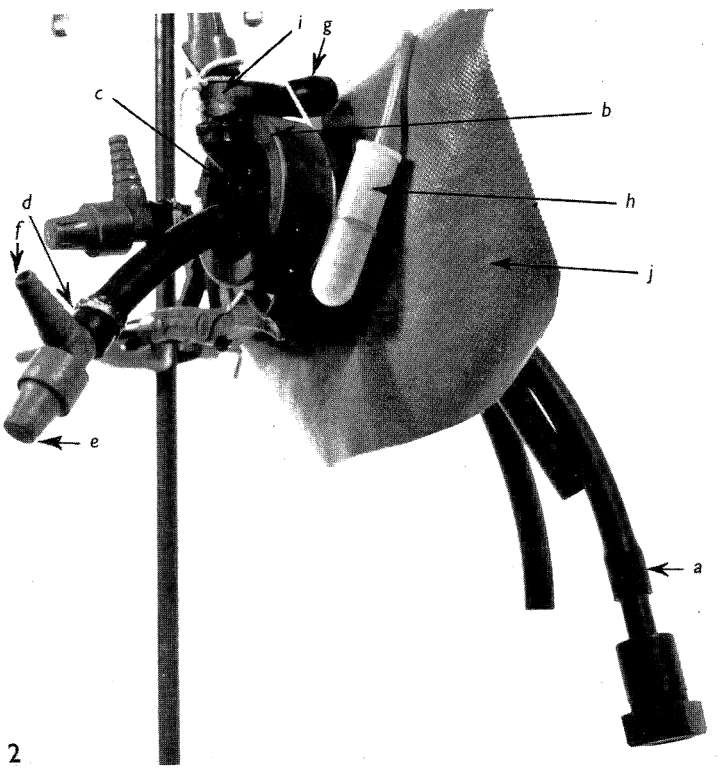

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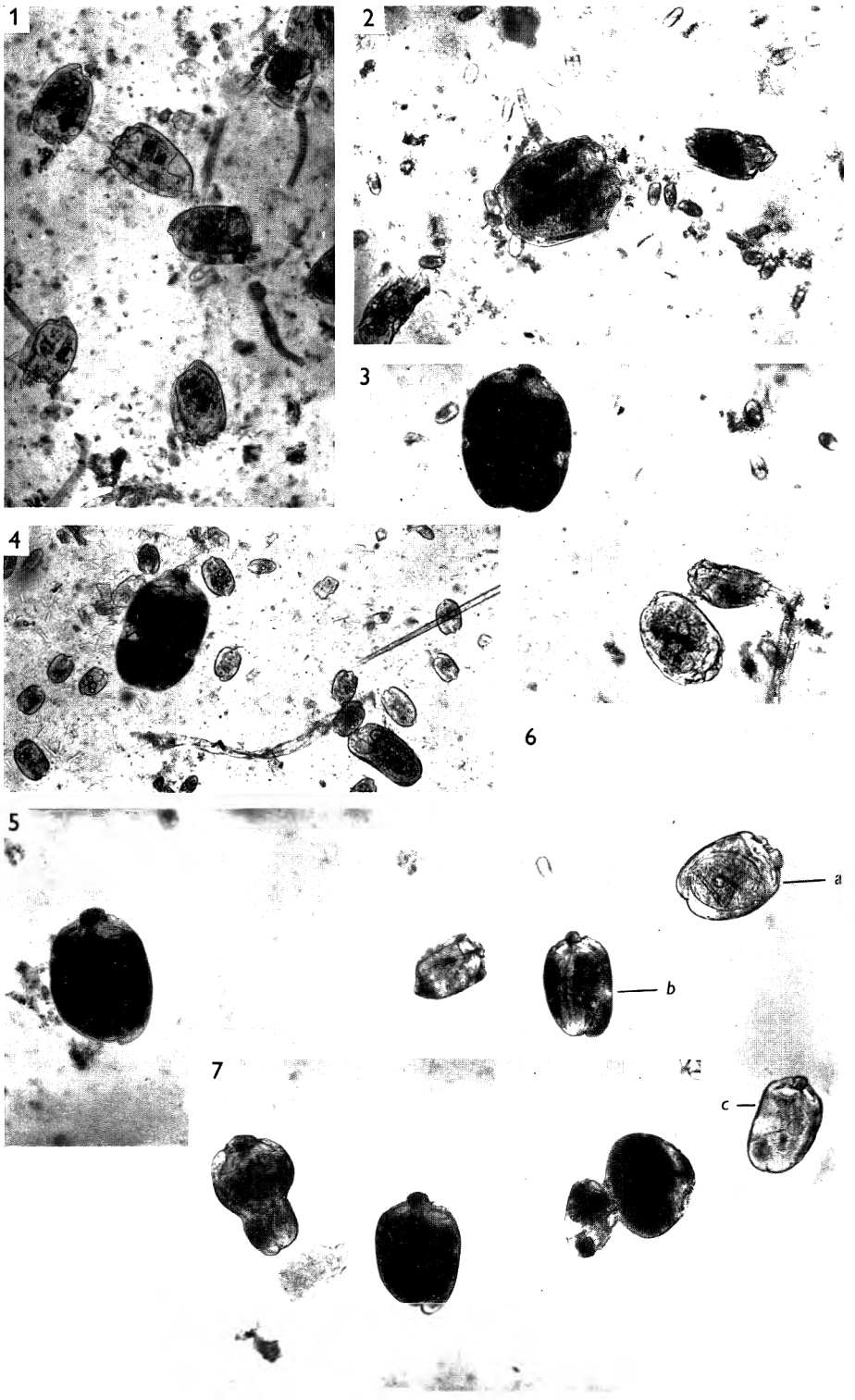

J. M. EADIE

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Electrophoresis of Proteins of 3 Penicillium Species on Acrylamide Gels

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SUMMARY

Soluble proteins extracted from mycelium of *Penicillium griseofulvum* were separated by acrylamide-gel electrophoresis. The pattern and overall intensity of the protein bands varied greatly with age of culture. A marked decrease in band intensity occurred at the time of exhaustion of the nitrogen source in shaken cultures, and at an earlier stage in static cultures; the total protein in whole or ultracentrifuged extracts did not decrease to the same extent. Changes in the pattern and intensity of protein bands during incubation occurred also in *P. chrysogenum* and *P. frequentans*. Mycelium of *P. griseofulvum* which was induced to sporulate in shaken culture yielded little protein as shown by electrophoresis at early stages of culture, in comparison with non-sporing mycelium. Each of these three Penicillium species could be distinguished by the protein pattern, which was reproducible and characteristic of the fungus at any particular stage of culture. The results indicate the need to determine the effects of age and conditions of culture when gel electrophoresis of mycelial proteins is used for taxonomic purposes.

INTRODUCTION

Gel electrophoresis is a valuable method for separating soluble protein fractions extracted from the mycelium of fungi and actinomycetes. Distinct and reproducible differences have been found between the protein patterns of related species and of strains within species (Chang, Srb & Steward, 1962; Clare, 1963; Durbin, 1966; Gottlieb & Hepden, 1966; Clare & Zentmyer, 1966). Changes in the age or conditions of culture appeared to have no appreciable effects on the relative intensities or positions of the protein bands (Clare, 1963; Durbin, 1966; Gottlieb & Hepden, 1966; Clare & Zentmyer, 1966). In the present investigation, however, the proteins of *Penicillium* spp. separated by electrophoresis were found to vary greatly according to the age and physiological condition of the mycelium.

METHODS

Organisms. The following fungi were used: *Penicillium griseofulvum* Dierckx (375); *P. chrysogenum* Thom (167); *P. frequentans* Westl. (733). The numbers refer to the culture collection of the Akers Research Laboratories.

Cultural methods. The fungi were grown from spore inocula, in shaken or in sta-

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tionary flasks, on a liquid glucose+ammonium sulphate+salts medium. Details of the cultural methods have been published (Bent & Morton, 1964).

Extraction and electrophoresis of mycelial proteins. Samples of mycelium were collected by filtration, washed twice with water and stored at $-15°$. Samples from shaken cultures (about 1 g. fresh weight) were placed in 9 ml. of cold 0·067 M-phosphate buffer (pH 7·0) and shaken at high speed with glass beads on a wrist-action shaker in a cold room (3°) for 30 min. Mycelial felts from static cultures were first broken up in buffer with a blender for 2 min. and then ground in a cooled glass tissue-grinder for 5 min.

The suspensions were centrifuged at 25,000 *g* for 30 min. Volumes of supernatant fluid equivalent to 1·25 mg. dry weight mycelium were submitted to disc electrophoresis on polyacrylamide gels. In general the procedure described by Ornstein & Davis (1962) and by Davis (1964) was followed, but samples were applied in 25% (w/v) sucrose solutions instead of in large-pore gels. Sometimes spacer gels were also omitted. The above methods were adopted largely for convenience, since no appreciable difference to the electrophoretic pattern was made by using different homogenisation techniques (grinding in a glass tissue-grinder, passage through a Hughes press, shaking with glass balls), by using different extraction fluids (water, phosphate buffer at pH 6·0 or 7·0, 0·25 M-sucrose in pH 7·0 buffer), by the presence or absence of large-pore sample or spacer gels, by conducting electrophoresis at 3° instead of at room temperature, by storing mycelium at $-15°$ for 2 weeks, by drying mycelium with acetone or by successive centrifugation of 1000 *g* supernatant fluids at 25,000 and 100,000 *g*. Electrophoresis patterns obtained with replicate portions of one protein extract, with replicate extracts from one culture, or with mycelial samples taken from separate but similar cultures of the same age grown on the same or on different occasions, were virtually identical when run in the same batch. Unpredictable minor variations in the relative mobility of bands sometimes occurred when one preparation was treated on different occasions under apparently the same conditions, although the overall pattern of bands was maintained. For this reason, samples were run in the same batch before detailed comparisons between different preparations were made.

Determination of protein. Total protein contents of mycelium and mycelial extracts were determined by the method of Lowry, Rosenbrough, Farr & Randall (1951), with bovine serum albumin (fraction V) as the standard.

The use of conventional methods (such as determination by the method of Lowry *et al.* or by measurement of α-amino-nitrogen after acid hydrolysis) to determine total protein in the gels or in extracts therefrom was precluded by the occurrence of high blank values, presumably due to the peptide links in the polyacrylamide. The amount of protein in the gels was estimated by determining directly the intensity of the Amido Black staining. After removal of the spacer gel, each small-pore gel was trimmed beyond the electrophoretic front to a suitable length, inserted into a glass tube from which its end protruded slightly and placed in a 4 cm. spectrophotometer cell so that its ends were just flattened against the walls of the cell. The cell was placed in a Unicam SP 500 spectrophotometer, with the gel aligned lengthwise in the light path, and the extinction at 600 mμ was measured against a blank gel. Standard gels were prepared from bovine serum albumin (fraction V). Extinction was related linearly to the amount of albumin or mycelial extract applied to the gels; readings from replicate gels were within 3% of the mean values.

RESULTS

The pattern and the overall quantity of soluble protein in electropherograms of extracts of *Penicillium griseofulvum* varied considerably during the course of shaken culture. The pattern shown in Pl. 1, fig. 1 A, was typical of mycelium in the exponential phase of growth (18–27 hr), and in the original gels some 16 bands could readily be distinguished. Shortly before the exhaustion of the nitrogen source (the limiting nutrient), which occurred at 28·5–29 hr, the pattern changed (Pl. 1, fig. 1 B); several new bands appeared, and there were marked changes in the intensities of many of the bands shown by earlier samples.

During the subsequent period of nitrogen starvation there was a very marked and persistent decrease in the overall amount of protein appearing in the gels (Pl. 1, fig. 1 C and D). Some bands decreased more than others, so that further changes in pattern could be distinguished. During this nitrogen-deficient stage of incubation, the hyphae continued to grow in length. When young (24 hr) mycelium was removed from the culture medium and shaken in fresh medium from which the nitrogen source was omitted, a similar rapid and large decrease in overall band intensity and similar changes in pattern took place. More concentrated extracts of older mycelium were used in an attempt to increase the band intensities, so that the patterns could be compared more easily with those given by younger cultures. However, the results were unsatisfactory since the rate of protein migration from the stronger extracts was much slower; after prolonged electrophoresis the bands became diffuse and were obscured by heavy background staining.

A comparable sequence of changes in the soluble proteins of *Penicillium griseofulvum* occurred when the fungus was grown in static culture. In this type of culture, however, changes were detectable at a relatively early stage, when only half of the available nitrogen had been consumed (170 μg. N/ml. remaining). Thus the gel shown in Pl. 1, fig. 1 F, representing mycelium grown in still culture for 50 hr (130 μg. N/ml. remaining), had a pattern intermediate between those of Figs. 1 A and B, but closer to that of Fig. 1 B, which was derived from a shaken culture in which the nitrogen source was almost exhausted. At later stages of static culture there occurred a further general decrease in band intensity and also changes in pattern similar to those observed in the shaken cultures.

Gels were also prepared from shaken cultures of *Penicillium griseofulvum* growing in a medium prepared with a crude commercial glucose instead of reagent grade glucose. The crude glucose contained factors which promoted the sporulation of this fungus in shaken culture (Armstrong, England, Morton & Webb, 1963); Pl. 1, fig. 1 E represents mycelium which was just starting to sporulate (the hyphae were highly branched and most of the tips bore incipient conidiophores). In general intensity of staining and in band pattern there was a resemblance to gels representing nitrogen-starved mycelium rather than to gels prepared from comparable young non-sporing cultures. This distinction between young mycelia of sporing and vegetative cultures was observed in several experiments. As the cultures aged, gels of sporing mycelium became in intensity and pattern more like those of non-sporing cultures of similar age.

The soluble proteins of *Penicillium chrysogenum* and *P. frequentans* (Pl. 1, fig. 2) changed with age of culture in much the same way as those of *P. griseofulvum*. Gels prepared from the three species at a particular time of incubation could be distinguished

easily from each other. An independent observer was able to sort nine gels which represented these species at three times of incubation (20, 28, 36 hr) correctly into groups representing each species, but only after the pattern of change, and particularly the general decrease in band intensity with age of culture, had been explained to him. When gels representing the middle stages of incubation were omitted, it was impossible to match the remaining gels.

The marked decrease in band intensity shown by gels prepared from older mycelium was unexpected, since earlier studies with *Penicillium griseofulvum* (Bent & Morton, 1964) had revealed that the total protein content of the mycelium underwent relatively small changes during shaken culture. The total protein contents of some electrophoresis gels were therefore compared with those of the mycelial extracts from which they were made (Table 1). Although determinations in gels and in extracts were made by different methods (see above), the large differences in the values for protein obtained from gels and from corresponding mycelial extracts suggest that even at early times of incubation only a small proportion of the protein present in whole extracts, or in ultracentrifuged extracts, was represented by the stained bands in the gels. This proportion which moved electrophoretically decreased greatly during shaken or static cultures, especially after exhaustion of the nitrogen supply (Table 1). Thus, under conditions of nitrogen starvation, there was a rapid and specific loss of those proteins which could enter gels and react with the Amido Black stain.

Table 1. *Amounts of protein in extracts and electrophoresis gels prepared from mycelium of Penicillium griseofulvum at two stages of culture*

Protein is expressed as mg./g. mycelial dry weight. Nitrogen was exhausted from the medium at 28·5–29 hr. (shaken culture) and 48–72 hr. (static culture). Extracts were centrifuged at 25,000 *g* for 30 min., and at 100,000 *g* for 75 min.

Type of culture	Shaken culture		Static culture	
Age of culture	24 hr	36 hr	48 hr	120 hr
Ammonia-N in medium (μg./ml.)	184	0	139	0
Total mycelial protein	557	403	—	—
Extracted protein in:				
(*a*) whole extract	298	208	323	271
(*b*) 25,000 *g* supernatant fluid	159	83·3	146	78·9
(*c*) 100,000 *g* supernatant fluid	—	—	123	50·0
Proteins in gels from:				
(*a*) whole extract	—	—	27·3	2·07
(*b*) 25,000 *g* supernatant fluid	28·4	1·04	18·6	1·60
(*c*) 100,000 *g* supernatant fluid	—	—	17·5	1·41

DISCUSSION

Changes in soluble protein content have been detected by various techniques in a wide range of organisms, including the fungi *Blastocladiella emersonii* (Cantino & Goldstein, 1962) and *Neurospora crassa* (Williams & Tatum, 1966), and reports of alterations in the amounts of individual enzymes in response to environmental factors are numerous. Nevertheless, in recent comparative studies of the soluble mycelial proteins of related species and strains of fungi the effects of the age and conditions of culture have appeared to be insignificant (see Introduction). The simplicity and sensitivity of the

acrylamide-gel technique, coupled with the suitability of the gels for long-term storage, may lead to its widespread adoption for taxonomic and diagnostic work on the fungi. It is becoming increasingly evident that electrophoretic separation of mycelial proteins can provide a useful criterion for distinguishing the genotype, and the present study provides another example of this. The results make it clear, however, that standardisation of cultural conditions and attention to the physiological age of the organism must not be overlooked in this type of work.

A striking feature revealed in the present work was the rapid and selective decrease in those proteins that migrated electrophoretically, which occurred in shaken cultures when the external supply of nitrogen was exhausted or when mycelium was transferred to nitrogen-deficient medium. In static cultures similar changes occurred at a relatively early stage, well before the nitrogen supply was used up. This probably reflected the morphological differences between the two types of culture. In shaken cultures the fungi grew as dispersed suspensions of hyphae which were in direct contact with the medium, whereas in static cultures the mycelia developed as surface felts, of which large proportions must have been relatively remote from the nutrient supply.

Submerged mycelium growing on a supplemented medium which induced sporulation gave electrophoretic patterns which resembled those of nitrogen-starved mycelium. In the absence of specific nutritional factors for sporulation, such as those provided here, nitrogen starvation is one essential condition of sporulation of this organism in submerged culture (Morton, England & Towler, 1958). The present results suggest that the factors in crude glucose may induce some of the metabolic effects normally brought about by nitrogen starvation, in young mycelium growing in the presence of assimilable nitrogen. The occurrence of a relatively low overall nitrogen content has been reported as another feature in common between young sporing mycelium and nitrogen-starved vegetative mycelium of *Penicillium griseofulvum* (Bent & Morton, 1964). Protein turnover at a high rate has been detected in the mycelium of *P. griseofulvum* under conditions of nitrogen deficiency (Bent, 1964), and it is possible that those proteins which appeared in the acrylamide gels were particularly liable to degradation and were not replaced by re-synthesis. Alternatively, such proteins may have aggregated or become bound into structures which were too large or carried an insufficient charge to be attracted into the gels, or which were not extractable from the mycelium. An apparent loss of the ethanol-soluble protein in *Escherichia coli* which occurred at the onset of sulphur starvation was shown to result from its interaction with intracellular polymetaphosphate that accumulated under this condition (Pine 1963) and not from its utilisation as a reserve material as first thought. Further investigations into the associational state of the mycelial proteins, and into the changes in the amounts and activities of specific enzyme proteins are warranted.

I wish to thank Mr D. H. W. Scott for his skilled technical assistance.

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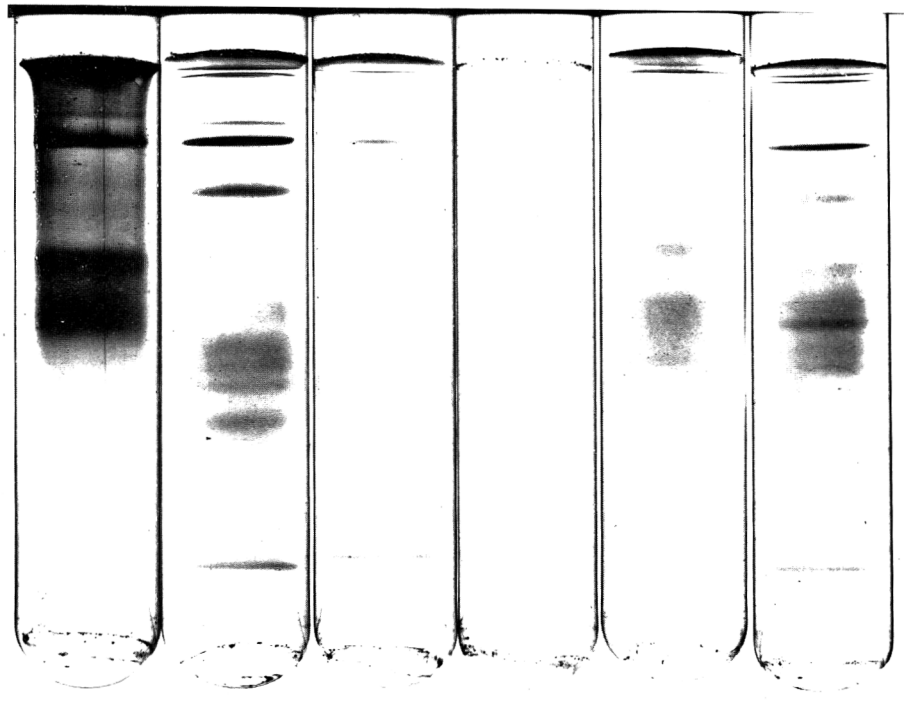
EXPLANATION OF PLATE

Fig. 1. Soluble mycelial proteins of *Penicillium griseofulvum*. A, B, C and D, from shaken cultures at 22, 28, 33 and 36 hr respectively after inoculation. E, from a 22 hr shaken culture induced to sporulate by factors in crude glucose. F, from a 50 hr static culture.

Fig. 2. Soluble mycelial proteins of *Penicillium chrysogenum* (A, B, C) and *P. frequentans* (D, E, F), from shaken cultures at 22 (A, D), 28 (B, E) and 36 (C, F) hr after inoculation.



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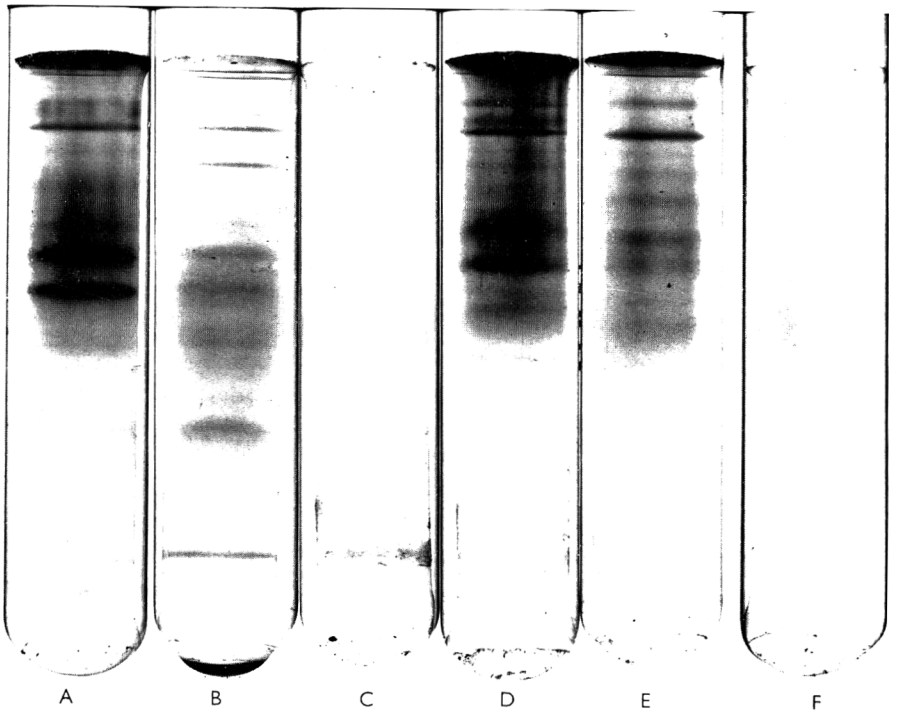

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Genetic Transformation in Pseudomonas

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(*Accepted for publication 5 May 1967*)

SUMMARY

Intraspecific and interspecific transformation in Pseudomonas has been investigated. Two isoleucine+valine requiring mutants of *Pseudomonas aeruginosa* have been transformed to prototrophy. By using gelatin liquifaction as a marker character it was observed that two strains of *P. fluorescens*, a phytopathogen *P. mangiferaeindicae* and a strain of *Pseudomonas* originally isolated from fish were transformable. When *P. solanacearum* was used as a donor, the transformation frequency of *P. mangiferaeindicae* was very high. DNA from fish Pseudomonas 1 was capable of transforming two strains of *P. fluorescens* and *P. mangiferaeindicae* in addition to fish Pseudomonas 21. When *P. fluorescens* was treated with the DNA from *P. aeruginosa* transformation frequency was lower; the capacity of pigment production, however, could not be transferred to any of the strains. The highest transformation frequency was obtained towards the end of the log phase of growth of the receptor bacteria, with a DNA concentration of about 10 μg./ml. Optimum temperature for transformation was 25–30° and agitation and starvation in diluted media increased the number of transformants. The significance of the observations has been discussed from the point of view of relationships among the strains and species studied.

INTRODUCTION

Taxonomy of the genus *Pseudomonas* is a matter of considerable controversy. The recognized manuals of bacterial classification describe more than one hundred species under this genus. The wealth of informations available has been critically examined by De Ley (1964) and Stanier, Palleroni & Doudoroff (1966) in two recent reviews. Adansonian analysis has decreased the number of species considerably. Marmur, Falkow & Mandel (1963) emphasized the importance of DNA base composition, DNA hybridization and genetic transformation as tools in bacterial taxonomy. Colwell & Mandel (1964), De Ley & Friedman (1965), Colwell, Citarella & Ryman (1965), Mandel, Weeks & Colwell (1965), De Ley, Park, Tijtgat & Ermengem (1966) and Mandel (1966) have reported the guanine+cytosine (GC) content of the DNA of 44 species belonging to this genus. Though most of the species reveal a GC content of 60–67% the range extends from 37 to 70%. The present communication records our observations about the transformability of twelve strains of Pseudomonas, with a view to understanding the inter-relationships of the strains studied.

METHODS

Organisms. The following cultures were used: five strains of *Pseudomonas aeruginosa* including two auxotrophic (isoleucine + valine) mutants, two phytopathogenic strains (*P. solanacearum*, *P. mangiferaeindicae*), one strain of *P. putrefaciens*, two strains of *P. fluorescens* and two other pseudomonads (fish Pseudomonas 1, 21)—which were originally isolated from freshwater fish and closely resembled *P. fluorescens*. These latter two strains differed from each other in the production of gelatinase, lipase and lecithinase, in the reduction of nitrate and in the formation of levan (Bullock, Snieszko & Dunbar, 1965). The two strains of *P. fluorescens* (ATCC 12633, and a strain obtained from Dr J. H. Darrell, henceforth be referred to as the Darrell strain) were incapable of liquefying gelatin (a property characteristic of *P. fluorescens*) thus confirming the observations of Bullock *et al.* (1965) and Dr J. H. Darrell (personal communication) concerning these two strains. In this respect these cultures resemble *P. putida* which Flügge (1886) considered to be a non-liquefying biotype of *P. fluorescens*. In absence of other details we shall refer to these strains as belonging to *P. fluorescens*. The strains of *P. mangiferaeindicae* and *P. putrefaciens* also did not liquefy gelatin, contrary to the description of these species in *Bergey's Manual of Determinative Bacteriology* (1957). All the five strains of *P. aeruginosa*, including the auxotrophic mutants, however, liquefied gelatin. This is in agreement with the observations of Stanier *et al.* (1966) that all strains of this species liquefy gelatin. The auxotrophic (isoleucium + valine-requiring) mutants *ilva A9* and *ilva C1* were originally obtained from the wild strain *P. aeruginosa* 78. *Pseudomonas solanacearum* had pronounced gelatinase activity. The source of the cultures is given in Table 1. All the cultures were purified twice before use.

Table 1. *Strains of Pseudomonas used and their sources*

Designation	Source
Pseudomonas aeruginosa 78 *P. aeruginosa ilva A9* *P. aeruginosa ilva C1*	Dr J. S. Loutit, Dept. of Microbiology, Medical School, University of Otago, Dunedin, New Zealand
P. aeruginosa LC-1 *P. aeruginosa* 215 *P. fluorescens*	Laboratory culture originally obtained from a Calcutta hospital. Dr J. H. Darrell, Dept. of Bacteriology, Post graduate Medical School of London, U.K.
P. fluorescens ATCC 12633 Fish Pseudomonas 1 Fish Pseudomonas 21	Dr G. L. Bullock, Eastern Fish Disease Laboratory, Leetown, W. Va., U.S.A.
P. solanacearum P 25 *P. mangiferaeindicae* P 24	Indian Type Culture Collection, New Delhi, India
P. putrefaciens NRRL B. 950	Dr W. C. Haynes, Agricultural Research Service, Northern Regional Research Laboratory, Peoria, Ill., U.S.A.

In addition to the Pseudomonas cultures the following organisms were also used in the transformation experiments: (i) *Bacillus subtilis* MB 294 (kindly supplied by Dr H. B. Woodruff of Merck, Sharp and Dohme, Rahway. N.J., U.S.A.), (ii) *Sarcina lutea* obtained from the Microbiology Laboratory of Dey's Medical Stores (Manufacturing) Private Ltd., Calcutta, (iii) *Azotobacter chroococcum* AZCF isolated from local soil samples in this laboratory (Sen & Sen, 1965).

Media. The cultures were usually grown in nutrient agar medium of the following composition: Lab Lemco, 10 g.; peptone, 10 g.; sodium chloride, 5 g.; glucose, 1 g.; agar, 20 g.; water to 1000 ml.; pH 7·2. Transformants obtained were maintained in nutrient gelatin medium containing nutrient broth with 15% (w/v) of gelatin (Difco) and without glucose. For maintenance of the auxotrophic mutants Morihara's (1964) medium having the following composition was used: Glucose, 1 g.; NH_4Cl, 1 g.; Na_2HPO_4, $12H_2O$ 1 g.; KH_2PO_4, 0·2 g.; $MgSO_4.7H_2O$, 0·05 g.; $CaCO_3$, 0·05 g.; isoleucine, 2 mg.; valine, 2 mg.; distilled water to 1000 ml. For scoring organisms transformed to prototrophy dilutions of the incubation mixture were plated on the minimal medium without valine and isoleucine.

Transformation. Competent bacteria were prepared according to the method of Stuy (1962) with slight modifications. Except in the experiments in which conditions were varied, 0·1 ml. of an 18-hr culture of a receptor strain was suspended in 0·8 ml. of normal saline and 0·1 ml. nutrient broth added, followed by shaking for 30 min. at 30°. The concentration of the recipient bacteria varied between 2 and $3{\cdot}5 \times 10^7$ bacteria/ml. After 'starvation' in this diluted medium for 30 min., 1·0 ml. of DNA solution obtained from the donor strain was added and the mixture incubated for another 30 min. at 30°, followed by treatment with DNase (Worthington Biochemicals Corporation, U.S.A.) 10 μg./ml. solution containing 5 μmoles $MgCl_2$/ml. The number of transformants/ml. was calculated by dilution plating in an appropriate media. In another set of experiments the DNA isolated from donor strains was first incubated with DNase for 5 min. at 37° and 1 ml. of this DNase-treated preparation was included in the transformation medium before the receptor bacteria were introduced. After incubation for 30 min. dilutions of the transformants, if any, were plated as described. Experiments were also done in which attempts were made to transform the receptor bacteria with its own DNA as also with the DNA isolated from such unrelated species as *Sarcina lutea*, *Bacillus subtilis* and *Azotobacter chroococcum*. In the experiments in which the optimum conditions were worked out, only the factor being studied was varied, all other conditions remaining unchanged.

Isolation of transforming DNA. The donor strains were grown in shake cultures for 18–20 hr and the bacteria were collected by centrifugation. Sometimes the donor strains were seeded on nutrient agar medium in Petri dishes, incubated for 18–20 hr and the bacteria harvested by gently scraping them from the surface of the agar. DNA was isolated and purified following the method of Marmur (1961) with slight modifications. Concentration of DNA was measured at 600 mμ in a Bausch and Lomb Spectronic-20 colorimeter by the diphenylamine method.

Genetic markers. In most of the experiments described here gelatin liquefying capacity was the marker used. In some of the experiments in which *Pseudomonas aeruginosa* was used as donor, formation of the green diffusible pigment was used as a marker character. For transformation of the auxotrophic (isoleucine + valine) mutants of *P. aeruginosa* to prototrophy the capacity to synthesize isoleucine + valine was used as a marker character.

Transformation assay procedure. Nutrient agar containing 0·4% (w/v) gelatin was used for scoring the transformants following Smith's modification of Frazier's method as described in the *Manual of Microbiological Methods* (1957). Dilutions of the reaction mixture were plated on this medium. The plates were incubated at 28–30° for 24–48 hr. The colonies were transferred to nutrient gelatin tubes and allowed to grow for 24–48 hr

at 28–30°. After incubation, each tube was kept in the refrigerator for several hours and solidification, if any, noted. Transformants capable of liquefying gelatin exhibited no solidification. When liquefaction was poor the incubation period was prolonged to 72 or 96 hr. The presence of gelatinase was also tested according to the following procedure. Ten ml. of a reagent containing 15 g. $HgCl_2$ dissolved in 100 ml. distilled water + 20 ml. conc. HCl when poured on the medium precipitated any unhydrolysed gelatin, giving a white opacity; absence of opacity surrounding a colony indicated its ability to hydrolyse gelatin. Similar results were also obtained by flooding the plates with a saturated solution of ammonium sulphate.

RESULTS

It was possible to transform six out of the eight strains used as receptor, using gelatin liquefaction or isoleucine + valine requirement as marker character. Neither of these characters, however, could be introduced in the receptor strains when they were treated with the DNA isolated from the same strain. When Pseudomonas receptor bacteria were treated with the DNA of such unrelated bacterial species as *Sarcina lutea*, *Bacilius subtilis* or *Azotobacter chroococcum*, the reported GC contents of which are 68–72, 39–45 and 57·5%, respectively (see Hill, 1966), there was no transformation with respect to the marker characters used, including gelatin liquefaction and pigment production. Incubation of the DNA of donor bacteria with DNase 10 μg./ml. at 37° for 5 min. before the treated DNA was applied to receptor bacteria resulted in a complete loss of its transforming ability in all the cases investigated.

Conditions for transformation

In preliminary experiments it was observed that transformability of the strains could be tested conveniently by using gelatin liquefying capacity as a marker character. Using a gelatin-liquefying strain fish Pseudomonas 1 as the donor, and a strain of *Pseudomonas fluorescens* ATCC 12633 as receptor the conditions for transformation were worked out. The age of the receptor bacteria having highest competency and the optimum concentration of donor DNA required for transformation were determined. The effects of the duration of incubation with transforming DNA, agitation of the incubation mixture, temperature and composition of the medium on transformation frequency were also studied. Under optimum conditions the frequency of transformation with these two strains was as high as 4·8%.

The formation of transformants depended considerably on the physiological state of the receptor strains. Accordingly, transformation experiments were made at different stages of growth. The highest number of transformants was obtained towards the end of the log phase; there was no further increase during the stationary phase. The occurrence of transformants increased with time and the transformation curve resembled considerably the growth curve, the maximum being attained at about 18 hr (Fig. 1).

When the DNA concentration was varied it was observed that transformation frequency increased with increasing concentration of DNA up to a certain limit, beyond which no further increase in the frequency was detected. Fig. 2 shows the response of the receptor strain *Pseudomonas fluorescens* ATCC 12633 to DNA preparations from fish Pseudomonas 1. The percentage of transformants increased linearly with concentration, the saturating concentration of transforming DNA being reached at about 10 μg. DNA/ml.

Transformation frequency increased rapidly with the time of exposure to DNA (Fig. 3). Within 5 min. 2·5% of the bacteria were transformed; the highest frequency was reached at 20 min.

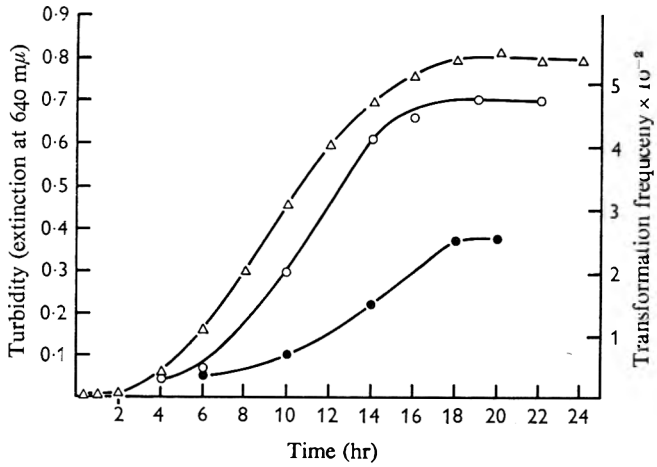

Fig. 1. Formation of transformants at different phases of growth. △——△, Growth curve of the receptor strain *Pseudomonas fluorescens* ATCC 12633; ○——○, transformation of *P. fluorescens* ATCC 12633 with fish Pseudomonas 1 DNA; ●——●, transformation of *P. fluorescens* ATCC 12633 with *P. aeruginosa* 215 DNA.

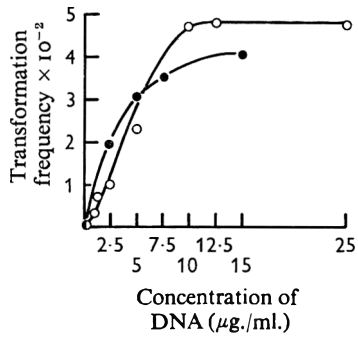

Fig. 2

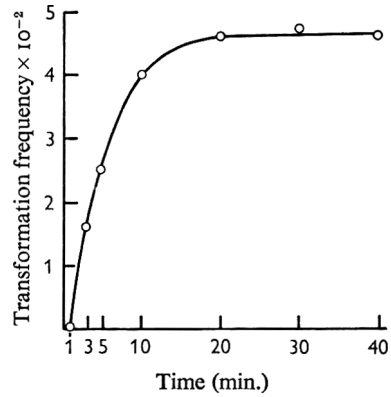

Fig. 3

Fig. 2. Effect of DNA concentration on transformation frequency. ○——○, Transformation of *Pseudomonas fluorescens* ATCC 12633; ●——●, transformation of fish Pseudomonas 21. Donor fish Pseudomonas 1.

Fig. 3. Effect of the time of incubation with DNA on transformation frequency of *Pseudomonas fluorescens* ATCC 12633 with the DNA of fish Pseudomonas 1.

The temperature range 25–30° was found to be the optimum. At temperatures lower than 20° the frequency was nil and at 37° it was negligibly small. Transformation frequency was enhanced when the amount of nutrient broth in the incubation medium was decreased. Highest frequency was observed when the broth was diluted about

10-fold. Agitation of the incubation mixture gave a higher frequency than in the stationary condition, when other conditions were unchanged.

Intraspecific and interspecific transformation

The possibility of intraspecific and interspecific transformation was examined by using the conditions found to be optimum for transformation. Recipient bacteria harvested before the termination of the logarithmic phase of growth were incubated with transforming DNA 10 μg./ml. for 30 min. at 30° in a 10-fold diluted nutrient broth with continuous stirring. Samples of the incubation mixture were then diluted in steps of 10 and plated on appropriate media.

Table 2. *Intraspecific transformation in Pseudomonas aeruginosa*

The marker characterizes (isoleucine + valine) requirement

Donor	Receptor	Transformation frequency (%)
P. aeruginosa 78	*P. aeruginosa ilva C1*	4·7
P. aeruginosa 78	*P. aeruginosa ilva A9*	1·4

Table 3. *Interspecific transformation in Pseudomonas*

Donor	Receptor	Marker	Transformation frequency (%)
P. aerugirosa LC-1	*P. fluorescens* (Darrell)	Gelatin liquefaction	0·73
P. aerugirosa LC-1	*P. fluorescens* ATCC 12633	Gelatin liquefaction	0·13
P. aeruginosa 215	*P. fluorescens* ATCC 12633	Gelatin liquefaction	2·25
Fish Pseudomonas 1	*P. fluorescens* (Darrell)	Gelatin liquefaction	0·16
Fish Pseudomonas 1	*P. fluorescens* ATCC 12633	Gelatin liquefaction	4·8
Fish Pseudomonas 1	Fish Pseudomonas 21	Gelatin liquefaction	4·0
Fish Pseudomonas 1	*P. mangiferaeindicae* P 24	Gelatin liquefaction	3·7
P. solanacearum P 25	*P. mangiferaeindicae* P 24	Gelatin liquefaction	11·1
P. aeruginosa LC-1	*P. fluorescens* (DARRELL)	Pigment production	No transformation
P. aeruginosa LC-1	*P. solanacearum* P 25	Pigment production	No transformation
P. aeruginosa LC-1	*P. mangiferaeindicae* P 24	Pigment production	No transformation
P. aeruginosa LC-1	Fish Pseudomonas 21	Pigment production	No transformation
P. aeruginosa LC-1	Fish Pseudomonas 1	Pigment production	No transformation
P. aeruginosa 215	*P. mangiferaeindicae* P 24	Pigment production	No transformation
P. aeruginosa 215	*P. solanacearum* P 25	Pigment production	No transformation
P. aeruginosa 215	Fish Pseudomonas 1	Pigment production	No transformation

Intraspecific transformation. The possibility of intraspecific transformation in *Pseudomonas aeruginosa* was investigated by using a wild-type strain, *P. aeruginosa* 78, as donor and two isoleucine + valine requiring mutants *ilva C1* and *ilva A9* as receptors. Both the strains could be transformed to prototrophy (Table 2) and the highest frequency was observed when recipient bacteria 16–18 hr old were used for transformation. The frequency recorded with the *ilva C1* strain was however much higher than that for the *ilva A9* strain.

Interspecific transformation. Choice of a suitable marker for the demonstration of interspecific transformation among the pseudomonads used posed a problem. Finally,

gelatin-liquefying capacity and pigment production were selected as markers. The results of the transformation experiments are summarized in Table 3.

Fish Pseudomonas 1 was a very effective donor, being capable of transforming both the strains of *Pseudomonas fluorescens*, *P. mangiferaeindicae* and fish Pseudomonas 21. Of the two strains of *P. fluorescens* used, *P. fluorescens* ATCC 12633 yielded a higher transformation frequency than the Darrell strain.

DNA from *Pseudomonas aeruginosa* was capable of transforming both the strains of *P. fluorescens*, but the gelatin-liquefying capacity only was incorporated; the property of pigment production could not be transferred to any other non-pigment producing strain. *P. mangiferaeindicae* was easily transformed by DNA preparations from *P. solanacearum*, the frequency of transformation being over 10% *P. mangiferaeindicae* was also transformed by DNA preparations from fish Pseudomonas 1; the frequency in this case was 3·7%.

A curious observation was the finding that though both the strains of *Pseudomonas fluorescens*, ATCC 12633 and the Darrell strain, were incapable of liquefying gelatin, when the former was transformed with the latter 2·0% of the transformants liquefied gelatin. Fish Pseudomonas 21 also developed gelatinase activity when treated with the DNA of another inactive strain, namely, *P. putrefaciens*. The latter, however, did not transform any of the strains of *P. fluorescens* (Table 4).

Table 4. *Development of gelatin-liquefying capacity in Pseudomonas strains by genetic transformation*

The donor and receptor strains were both incapable of gelatin liquefaction.

Donor	Receptor	Transformation frequency (%)
P. fluorescens (DARRELL)	*P. fluorescens* ATCC 12633	2·0
P. putrefaciens NRRL B-950	Fish Pseudomonas 21	0·1
P. putrefaciens NRRL B-950	*P. fluorescens* (DARRELL)	nil
P. putrefaciens NRRL B-950	*P. fluorescens* ATCC 12633	nil

DISCUSSION

The frequency of transformation depends in a large measure on the similarity of DNA base composition, pairing compatibility of donor and receptor DNAs, the degree of homology within the marker locus and in the rest of the DNA molecule, host-induced modifications, the permeability of the recipient cell membrane to donor DNA and the quality of the DNA preparation used for transformation, in addition to the conditions used for the transformation experiments. The significance of the transformation frequency values for determination of interrelationships must be assessed with these limitations in view. The transformation of the auxotrophic mutants of *Pseudomonas aeruginosa* to prototrophy and the relatively high frequency observed in the transformation of gelatinase $^-$ to gelatinase $^+$ strains belonging to different species indicate that both intraspecific and interspecific transformation is possible at least among the species of Pseudomonas studied here. All the four gelatin-liquefying strains used as donor in the transformation experiments (*P. aeruginosa* LC-1, *P. aeruginosa* 215, fish Pseudomonas 1, *P. solanacearum*) have transferred this property to non-liquefying strains belonging to *P. fluorescens*, *P. mangiferaeindicae* and fish Pseudomonas 21. The frequency varied from 0·13% to 11·1%; in more than half of

the cases it was above 2%. The pigment-producing capacity of *P. aeruginosa*, however, was not transferred to any of the strains used.

The recipient strains apparently vary considerably in their capacity to integrate the piece of DNA responsible for the marker character. Thus while the wild strain *Pseudomonas aeruginosa* 78 transformed the two isoleucine+valine requiring mutants (*ilva A9*, *ilva C1*) to prototrophy, the efficiency of transformation of the former was only one-third that of the latter. The same observation also holds true for the two strains of *P. fluorescens* used, the Darrell strain and ATCC 12633.

If transformability of a strain with the DNA of another is an indication of the interrelationship of the two, then *Pseudomonas aeruginosa* would appear to be related to *P. fluorescens* since both the strains of *P. aeruginosa* (LC-1, 215) were capable of transforming the Darrell and ATCC 12633 strains of *P. fluorescens*. These two strains of *P. fluorescens* were also transformable with the DNA of fish Pseudomonas 1 which also transformed fish Pseudomonas 21 with a high frequency. Fish Pseudomonas 1 and 21 thus seem to be related with each other and with *P. fluorescens*. On the basis of biochemical and growth characteristics Bullock *et al.* (1965) concluded that fish Pseudomonas 1 and 21 were 'closely related to or identical with *P. fluorescens*'.

Fish Pseudomonas 1 also transformed the phytopathogen *Pseudomonas mangiferaeindicae* with a frequency of 3·7%. When another phytopathogen *P. solanacearum* was used as donor the frequency rose to 11·1%, the highest recorded for any of the Pseudomonas strains. The two phytopathogens thus are probably very closely related. If fish Pseudomonas 1 is a biotype of *P. fluorescens* then *P. mangiferaeindicae* and *P. solanacearum* would appear to be near relations of such a biotype. Many phytopathogenic pseudomonads are fluorescent, differing from each other in their capacity to attack specific hosts; they have many characters in common and Starr (1959) and Stanier *et al.* (1966) have questioned the validity of many phytopathogenic species. There are several reports of the failure of phage preparations to distinguish between many phytopathogenic and saprophytic pseudomonads, implying a close interrelationship among them. Many phytopathogenic pseudomonads have also been reported to be antigenically related.

Stanier *et al.* (1966) published extensive observations on the physiological, biochemical, nutritional and growth characteristics of 267 strains of aerobic pseudomonads and have clearly brought out the similarities and dissimilarities of the different species and biotypes From an Adansonian analysis of several Pseudomonas strains Colwell & Mandel (1964) found that the strains of Pseudomonas used by them revealed a high degree of mutual similarity suggesting a species level relationship. Some of the *Pseudomonas fluorescens* strains 'clustered' within the *P. aeruginosa* group. Based on maximal intergroup S-values the relationship between the *P. fluorescens* group and the *P. solanacearum* group was found to be about 67%.

The DNA base composition of the strains used here is not known. It may be mentioned that the GC content of the DNA of seven biotypes of *Pseudomonas fluorescens* A, B, C, D, E, F and G has been reported to lie between 58·2 and 64·2% and that of *P. putida* biotypes between 59 and 63% (Mandel, 1966). The similarity of base composition among the *P. fluorescens* biotypes particularly A, B, C, F and G and between these biotypes and *P. putida* is thus considerable, suggestive of the possibilities of successful transformation among them. *P. aeruginosa* DNA is slightly richer in guanine and cytosine, the GC content being 67·3–68·4%. There are reports in the literature

that transformation is possible among strains which differ significantly in their DNA base composition, if the nucleotide sequences are common to the strains and can be recognized and incorporated in the recipient genome (Marmur *et al.* 1963). From DNA hybridization experiments De Ley *et al.* (1966) determined the degree of homology between different species of Pseudomonas and have numerically fixed their relatedness. The DNA homology of *P. putida* and *P. aeruginosa* relative to *P. fluorescens* DNA was 70·5 and 63%, respectively. The corresponding taxonometric similarity between these species as calculated by Lysenko (1961) was 78 and 72%. The present results obtained from the transformation experiments are thus in good agreement with the tentative conclusions drawn earlier about interrelationship among the pseudomonads from comparative biochemical and morphological studies of the strains, Adansonian analyses and DNA base composition and hybridization data.

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A Taxonomic Study of *Acinetobacter* and Related Genera

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CONTENTS

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SUMMARY

A computer survey was made to find the relationships of 120 strains of Gram-negative or Gram-variable non-motile coccoid rods, isolated by the author and formerly called *Achromobacter*. They were compared with 75 named strains of various Gram-negative genera, on the basis of morphological properties and biochemical tests. A recently developed similarity coefficient was used and strains were sorted into phenons by single linkage. Most of the strains were grouped at 72·5% similarity into one large phenon. Strains outside this phenon were: *Pseudomonas aeruginosa* (2), *P. fluorescens* (3), *P. saccharophila* (1), *P. putrefaciens* (1), *Escherichia coli* (1), *Cellulomonas biazotiea* (1), *Arthrobacter globiforme* (1). These were differentiated from the 72·5-phenon by various combinations of the following characters which were nearly always negative for strains within the phenon: fermentative metabolism of sugars, pigmentation, production of reducing compounds from gluconate, alkalinity from arginine, hydrolysis of starch or proteins.

Within the 72·5-phenon, five smaller phenons were distinguished, most of them grouped at 82·5% similarity. The strains in each of these phenons and the characters possessed by a majority of strains and useful for the differentiation of these phenons were:

Phenons 1 and 5 were Gram-negative motile rods, usually peritrichous.

Phenon 1: *Agrobacterium* sp. (2), *Achromobacter hartlebii* (1), *Agr. tumefaciens* (1). Acid was produced oxidatively from glycerol, sucrose, fructose, lactose, arabinose, xylose, galactose and glucose; H_2S produced in Kligler medium; the Kovacs oxidase test positive; nitrate reduced; growth on Simmons medium and on Paton medium; no growth on digest agar at 0° or 37°; not sensitive to 2·5 i.u. of penicillin.

Phenon 5: *Alcaligenes faecalis* (3), *A. viscosus* (1), *A. denitrificans* (1), *A. bookeri* (1), *Alcaligenes* sp. (2). Acid not produced from sugars; oxidase-positive; nitrate reduced; growth on Simmons medium; no growth on Paton medium; growth on digest agar at 37° but not at 0°; litmus milk became alkaline; not sensitive to 2·5 i.u. of penicillin.

Phenons 2, 3 and 4 were Gram-negative coccoid rods, cocci or short rods, often in pairs.

Phenon 2: *Bacterium* (or *Acinetobacter* or *Achromobacter*) *anitratum* (10), *Achromobacter lacticum* (2), *Herellea* sp. (8), *Diplococcus mucosus* (6), *Moraxella lwoffi* (1), MJT isolate (1). Acid not produced from glycerol, sucrose or fructose; oxidative acid production from lactose, arabinose, xylose, galactose and glucose; oxidase-negative; nitrate not reduced; growth on Simmons medium and on Paton medium; growth on digest agar at 37° but not at 0°; litmus milk became acid; not sensitive to 2·5 i.u. of penicillin.

Phenon 3: *Achromobacter* sp. (1), MJT isolates (22). Acid not produced from glycerol, sucrose or fructose; oxidative acid production from lactose, arabinose, xylose, galactose and glucose; oxidase-positive; nitrate reduced; no growth on Simmons or Paton media; growth on digest agar at 0° but not at 37°; no pH change in litmus milk; sensitive to 2·5 i.u. of penicillin.

Phenon 4: *Mima* spp. (7), *Achromobacter venenosum* (1), *Alcaligenes viscosus* (2), *Moraxella lwoffi* (4), *Achromobacter* sp. (2), *Neisseria catarrhalis* (1), MJT isolates (93). Acid not produced from sugars; no growth on Simmons medium; growth on digest agar at 0° but not at 37°; no pH change in litmus milk; sensitive to 2·5 i.u. of penicillin. Other characters variable.

Phenons 4i, 4ii, 4iii: small phenons grouped at about 92·5% similarity within Phenon 4. In properties other than sugar oxidation, Phenon 4i resembled Phenon 3, and Phenons 4ii and 4iii resembled Phenon 2, but not so closely.

The minimum inhibitory concentration of penicillin was estimated for selected strains of Phenons 2, 3 and 4, and varied from < 0·1 to > 100 i.u./ml. Strains of Phenons 3 and 4i were very sensitive, those of Phenons 2 and 4iii were resistant, and those of 4ii were intermediate or resistant. Ungrouped strains of Phenon 4 varied from very sensitive to resistant.

It was concluded, on the basis of these results and the data of Mandel & Thornley (1967) on DNA composition, that the non-motile coccoid rods in Phenons 2, 3 and 4 should be placed in a separate genus from other strains in the survey. *Acinetobacter* Brisou & Prévot is the most suitable generic name, and strains in Phenon 2 correspond to the type species, *Acinetobacter anitratus*. The relationships of Phenon 3 and the sub-divisions of Phenon 4 need further study, and it is not suggested that they should be given specific rank at present.

Phenons 2, 3 and 4 included strains formerly called *Alcaligenes viscosus* and *Achromobacter*; their classification as *Acinetobacter* entails division of the genera *Alcaligenes* and *Achromobacter*. The genus *Alcaligenes* should be retained for strains resembling the motile, peritrichously flagellate type species, *A. faecalis*, represented in this study in Phenon 5. It is suggested that the genus *Achromobacter* also should be reserved for any motile peritrichous strains which may prove suitable for inclusion. The few strains in this survey which had these properties were not very similar to each other, and the borderline between these strains and *Agrobacterium* was not clear. The strain ATCC 15716, proposed as representative of the type species *Achromobacter liquefaciens* (Tulecke *et al.* 1965) was unlike all other strains in the survey; ATCC 15716 fermented sugars and contained a large proportion of Gram-positive cells, and may be closer to a Gram-positive genus than to those studied here.

INTRODUCTION

This taxonomic study was prompted by the difficulty of finding a satisfactory way of classifying Gram-negative aerobic non-motile cocci or coccoid rods which were isolated from poultry. In previous papers they have been referred to as *Achromobacter* (Thornley, 1960*a*; Thornley, Ingram & Barnes, 1960). Although their properties did not disagree with the extremely vague description of the genus in *Bergey's Manual* (1957), this classification was not satisfactory because these strains did not resemble the type species, *Achromobacter liquefaciens*, in motility or other properties, nor did they correspond to any other species listed in *Bergey's Manual* (1957).

The genus *Alcaligenes*, as described in *Bergey's Manual* (1957), appeared to be equally appropriate for all except a small proportion of the strains, which produced

acid by oxidation of glucose. A new genus, *Acinetobacter*, was proposed by Brisou & Prévot (1954) for non-motile bacteria previously classed as *Achromobacter* or *Alcaligenes*, and this also seemed a possible place for the isolates from poultry. However, it seemed doubtful whether separation of a genus on the one property of non-motility was justifiable.

This confused situation was reflected in the literature, where bacteria with similar properties, isolated from poultry, were described as 'similar in many respects to *Alcaligenes viscosus*' (Ayres, Ogilvy & Stewart, 1950) or as belonging to the *Achromobacter–Alcaligenes* group (Nagel *et al.* 1960). Similar isolates from fish (Shewan, Hobbs & Hodgkiss, 1960) and from meat (Buttiaux, 1961) were designated *Achromobacter* by these authors. However, the unsatisfactory nature of this genus, and particularly the lack of any strains corresponding to the type species, has been emphasized by Ingram & Shewan (1960).

Morphological features resembling those of the poultry isolates have also been mentioned in descriptions of *Bacterium anitratum*, *Mima* spp., *Herellea* spp., *Diplococcus mucosus*, *Moraxella* spp. and *Neisseria* spp. and it was not clear from the literature to what extent some of these groups were closely related or even synonymous.

The main object of the present work was therefore to compare the 'Achromobacter' strains isolated from poultry with named strains of all the genera mentioned. For this the methods of numerical taxonomy proposed by Sneath (1957) seemed particularly suitable. A preliminary study (Thornley, 1960*b*), by the use of these methods, showed that *Pseudomonas* strains, including both pigmented and non-pigmented isolates from poultry and from culture collections, could be grouped together and completely separated from 25 'Achromobacter' isolates from poultry. Among the 'Achromobacter' strains three small subgroups appeared, with a fairly low similarity to each other. In the present work it was intended to check the existence of these groups when a much larger number of strains was compared.

One feature of Sneath's (1957) method was its emphasis on positive characters in calculating similarity coefficients; where two strains compared were both negative in any character this did not contribute to the similarity coefficient. It is possible that this caused the much higher similarity found within the group for *Pseudomonas* strains than for 'Achromobacters' (Thornley, 1960*b*), because the 'Achromobacters' gave fewer positive results. For the present work a new coefficient of similarity, described by J. C. Gower (to be published), was used; this method allows the inclusion of 'negative matches' where it is thought appropriate.

METHODS

Section 1. Bacteriological methods

1.1. *Sources of strains*

The named strains obtained from national or private collections are listed in Table 1. Besides the genera thought to be possibly related to 'Achromobacter', strains of some other well-known Gram-negative genera (e.g. *Escherichia*, *Aeromonas*, *Pseudomonas*) were included for reference purposes. Seventy-five named strains were included in the computer survey, and two additional strains were obtained and studied later.

Strains of 'Achromobacter' isolated from poultry carcasses by the author are designated MJT followed by the experiment number, either F4 or F5, then by the

Table 1. *Named strains from culture collections included in the survey*

Strain no.	Designation and source
1	*Achromobacter anitratus* NCTC 8102 (B5W of Stuart, Formal & McGann, 1949)
2	*A. hartlebii* NCIB 8129 (ATCC 365; NRRL B-2392)
3	*A. lacticum* NCIB 8208 (NRRL B-551)
4	*A. lacticum* NCIB 8209 (NRRL B-552)
5	*A. venenosum* NCIB 9022
6	*Achromobacter* EB/F64/100 (Barnes)
7	*Achromobacter* CB 11 (Holding)
8	*Achromobacter* 7 A 14 (Holding)
9	*Achromobacter* A 16 (Moore)
10	*Achromobacter* 131 (Shewan)
11	*Achromobacter* 138 (Shewan)
12	*Achromobacter* 25 A 2 (Sulzbacher)
13	*Acinetobacter anitratum* NCIB 9019 (strain 64 of Brisou, 1957)
14	*Aeromonas formicans* (strain 18 of Eddy, 1960)
15	*A. liquefaciens* (*Pseudomonas hydrophila*) NCTC 7812 (Eddy, 1960)
16	*Agrobacterium tumefaciens* NCIB 8150 (ATCC 4720)
17	*Agrobacterium* LK 10 (Holding)
18	*Agrobacterium* ZH 1 (Holding)
19	*Alcaligenes faecalis* NCTC 415
20	*A. faecalis* NCTC 8764
21	*A. faecalis* NCTC 655
22	*A. faecalis* NCTC 8769
23	*Alcaligenes* sp.
24	*Alcaligenes bookeri* NCIB 8155
25	*A. denitrificans* NCTC 8582 (Leifson & Hugh, 1954)
26	*A. viscosus* NCTC 3233
27	*A. viscosus* NCIB 8154 (ATCC 9036)
28	*A. viscosus* NCIB 8596
29	*Alcaligenes* CS 8 (Holding)
30	*Alcaligenes* CS 11 (Holding)
31	*Arthrobacter globiformis* NCIB 8602 (ATCC 4336)
32	*Bacterium anitratum* B 5 (Ontario) strain 705 (Schaub)
33	*B. anitratum* B 7 (Ontario) strain EDDY (Schaub)
34	*B. anitratum* B 9 (Ontario) strain BIOL 2 (Schaub)
35	*B. anitratum* B 10 (Ontario) (strain 90 of Schaub & Hauber, 1948)
36	*B. anitratum* B 11 (Ontario) (strain 93 of Schaub & Hauber, 1948)
37	*B. anitratum* B 16 (Ontario) NCIB 9293 (strain B5 W3 of Ferguson & Roberts, 1950)
38	*B. anitratum* B 24 (Ontario) (strain B5 W72 of Ferguson & Roberts, 1950)
39	*B. anitratum* B 25 (Ontario) NCIB 9301 (strain B5 W99 of Ferguson & Roberts, 1950)
40	*Cellulomonas biazotea* NCIB 8077 (ATCC 486)
41	*Diplococcus mucosus* 169 (Klinge)
42	*D. mucosus* 019 (Klinge)
43	*D. mucosus* SO 1472/61 (Klinge)
44	*D. mucosus* SO 1506/61 (Klinge)
45	*D. mucosus* E 2241/60 (Klinge)
46	*D. mucosus* E 8743/60 (Klinge)
47	*Escherichia coli* NCTC 9001
48	*Herellea* Z 6 (Goldberg)
49	*Herellea* Z 7 (Goldberg)
50	*Herellea* Z 8 (Goldberg)
51	*Herellea* 5937 (King)
52	*Herellea* 5939 (King)
53	*Herellea* 5942 (King)
54	*Herellea* 5944 (King)
55	*Herellea* 6009 (King)
56	*Mima* Z 1 (Goldberg)
57	*Mima* Z 2 (Goldberg)
58	*Mima* Z 3 (Goldberg)

Table 1 (*contd*)

Strain no.	Designation and source
59	*Mima* z 4 (Goldberg)
60	*Mima* 5902 (King)
61	*Mima* 5936 (King)
62	*Mima* 5979 (King)
63	*Moraxella lwoffi* NCTC 5866
64	*M. lwoffi* NCTC 5867
65	*M. lwoffi* NCTC 7976
66	*M. lwoffi* 950/56 (Klinge)
67	*M. lwoffi* 148/57 (Klinge)
68	*Neisseria catarrhalis* NCTC 3622
69	*Pseudomonas aeruginosa* NCTC 6750
70	*P. aeruginosa* NCTC 2000
71	*P. fluorescens* NCTC 4755
72	*P. fluorescens* NCIB 3756
73	*P. fluorescens* 23/5 (Rhodes) (NCTC 10038; NCIB 9046; ATCC 13525)
74	*P. putrefaciens* NCIB 8615
75	*P. saccharophila* NCIB 8570 (ATCC 9114; NRRL B-1492)
*	*Achromobacter* sp. NCIB 9650 (strain SKERMAN 381 of Hendrie *et al.* 1964, see also Skerman *et al.* 1958)
*	*Achromobacter liquefaciens* ATCC 15716. (Tulecke *et al.* 1965)

* These two strains were studied after the computer survey was made.

Sources of strains

Dr E. M. Barnes, Low Temperature Research Station, Cambridge.

Dr B. P. Edey, Low Temperature Research Station, Cambridge.

Professor H. S. Goldberg, Department of Microbiology, University of Missouri, Columbia, Missouri, U.S.A. Strains of *Herellea* and *Mima* from human and animal sources.

Dr A. J. Holding, The Edinburgh School of Agriculture, Edinburgh 9. Strains of *Achromobacter*, *Agrobacterium* and *Alcaligenes* from soil.

Dr E. O. King, Communicable Disease Center, Public Health Service, U.S. Department of Health, Education and Welfare, Atlanta, Georgia, U.S.A. Strains of *Herellea* and *Mima* from human sources.

Dr K. Klinge, Universität des Saarlandes, Medizinische Fakultät, Institut für Hygiene and Mikrobiologie, Homburg, Germany. Strains of *Moraxella lwoffi* received from Dr Klinge were isolated by Dr H. Flamm, Hygiene-Institut der Universität, Wien, Austria, and strains of *Diplococcus mucosus*, were isolated from human sources by Dr F. Legler, Staatliche Bakteriologische Untersuchungs-Anstalt, Erlangen, Germany.

Dr H. B. Moore, Donald N. Sharp Memorial Community Hospital, San Diego, California, U.S.A.

Ontario Department of Health Laboratory, 360 Christie Street, Toronto 4, Ontario, Canada. Strains of *Bacterium anitratum* originally received from Dr I. Schaub, Johns Hopkins Hospital, Baltimore, Ma. and from Michigan Department of Health, Lansing, Michigan, U.S.A.

Dr M. E. Rhodes, Department of Botany, Coleg Prifysgol Cymru, Aberystwyth.

Dr J. M. Shewan, Torry Research Station, Aberdeen. Strains of *Achromobacter* from fish.

Dr W. L. Sulzbacher, Bureau of Animal Industry, Agriculture Research Administration. U.S. Department of Agriculture, Beltsville, Maryland, U.S.A.

strain number. Sixty-seven F4 isolates and 53 F5 isolates were examined, and this included the 25 strains previously used (Thornley, 1960*b*). The experiments from which these strains were derived were described by Thornley *et al.* (1960), together with the differential tests used to separate these bacteria from Pseudomonadaceae and Enterobacteriaceae. Several factors may be of importance in the present study. The poultry-production plants supplying chicken for the two experiments differed in processing methods, so that the respective microfloras might well differ. Most isolates were obtained from carcases after storage at chill temperatures (1–3°), although some were from initial samples. The effects of irradiation and chlortetracycline treatment

in delaying bacterial multiplication were studied, and some isolates were from chicken which had received one or both of these treatments.

1.2. *Temperature of incubation*

In general, tests were incubated at 20° except for tests of ability to grow at 0° and 37°. Some of the bacteria which originated from human sources grew very slowly at 20° and these were incubated at 25° with the same times for reading results and finishing the tests. This also applied to six strains isolated from soil and provided by Dr A. J. Holding.

1.3. *Basal media*

Abbreviations used are as follows: DA, digest agar (Mackie & McCartney, 1950); YEB and YEA, yeast extract broth and agar, consisting of digest broth with 0·3% yeast extract added; HIA, Difco Heart Infusion agar; FA, medium B (for maximum fluorescin production) of King, Ward & Raney (1954).

These media were used as indicated below and in Table 3. In general, the method used was standard for any test, except for a few which were used as preliminary screening tests (observations on colonies, oxidase test, antibiotic sensitivity).

Stock cultures were kept at 5° in $\frac{1}{4}$ oz. (bijou) bottles, on slopes of Collins agar (Barnes & Thornley, 1966); a selection of these cultures has since been freeze-dried. Slopes of HIA medium in 1 oz. bottles were grown and used for inoculating some test media, while for others, suspensions in 0·85% (w/v) NaCl solution made from these slopes, or peptone water cultures inoculated from them, were used.

1.4. *Morphological observations*

Organisms were streaked for single colonies on DA or YEA (Expt. F4) or HIA (Expt. F5) media, and the plates incubated for 2 days at 20° or 25°. These were used for the observations of colony form (Table 2), except for fluorescence which was observed on FA under ultraviolet radiation.

A 24-hr culture in YEB was examined by phase contrast for motility and shape, and then Gram-stained by Kopeloff & Beerman's modification (Mackie & McCartney, 1950). Gram-stains were also made on organisms from colonies after incubation for 2 days. The term 'Gram-variable' (Table 2) was used when some organisms in the smear stained Gram-positive and others Gram-negative; this tended to happen particularly with freshly isolated strains.

Organisms were not measured, but their shape was recorded after microscopic observation as follows, length:width ratio greater than 3:1 rods, 2–3:1 short rods, 1–2:1 coccoid rods, 1:1 cocci. Often several shapes were present in one preparation, and sometimes the three observations made for each strain were not all the same. From the information collected the shape which seemed most prevalent for each strain was selected and used for the computer coding.

Flagellar staining by Rhodes's (1958) method was done for most of the motile strains.

A few strains were examined by the electron microscope, with a negative-staining technique (Thornley & Horne, 1962) to reveal flagella, fimbriae and general morphology. For one strain, thin sections were also prepared, after fixation by the method of Ryter & Kellenberger (1958).

Table 2. *Computer coding of morphological observations*

Colony form		Cell morphology	
Computer coding*	Tests and levels	Computer coding*	Tests and levels
D 1	Diffusible pigment	A 0	Gram negative
D 1	Fluorescence on FA (seen under UV)	1	Gram variable
A 0	Colour white or cream	2	Gram positive
1	Colour yellow or orange	A 0	Rods
2	Colour brown	1	Short rods
A 0	Consistency butyrous	2	Coccoid rods
1	Consistency mucoid	3	Cocci
2	Consistency gluey	A –	No grouping of cells
3	Consistency coherent	0	Pairs
A 0	Edge entire	1	Short chains
1	Edge spreading or irregular	2	Long chains
A 0	Surface smooth	3	Clumps
1	Surface rough or dull	A 0	Non-motile
2	Surface wrinkled, pitted or irregular	1	Motile
		D 1	Flagella polar
		D 1	Flagella lateral

* D, dichotomies; A, alternatives (qualitatives).

1.5. *Biochemical and physiological tests*

The methods used, and the computer coding of the results, are listed in Table 3, and will not be described further, except for some details of Hugh & Leifson's test.

1.5.1. *Hugh & Leifson (1953) test*

The basal medium was made according to Hugh & Leifson's original formula, and sugar solutions were added after sterilization to a final concentration of 1%. Fructose and sucrose were Seitz-filtered, and other sugars as 15% (w/v) aqueous solutions were autoclaved at 115° for 20 min.

A preliminary test with basal medium + glucose in open and sealed bottles showed whether the metabolism of glucose was fermentative or not. When it was fermentative, both open and sealed bottles of the other sugars were tested. When not fermentative, only open bottles were used, although a further check was made by inspecting these after 2 or 3 days of incubation. Strains which oxidized a sugar then usually showed a layer of acid medium at the top of the bottle only; where there was acid throughout, at first inspection, the test was repeated with a sealed bottle to make sure that the reaction was not fermentative. An open bottle of the basal medium without additions was always inoculated, so that changes in reaction due to metabolism of the peptone were not confused with those caused by metabolism of the sugar.

The results were classed as 'fermentative' where acid was produced in the sealed bottle. Gas production was also noted, but this character was only coded for glucose metabolism, since when produced at all, it was usually produced with every sugar tested. Failure to produce acid was coded as 'inert'. Where acid was produced in the open tube only, developing from the surface downwards, the result was classed as 'oxidative', provided that the open bottle became completely yellow (pH 6 or less) within 14 days. This limit was chosen to exclude extremely feeble and slow acid production, which caused a slight change in the indicator, often at the top of the

Table 3. *Tests used, and computer coding of results*

Test	Incubation period (days)	Computer coding*	Level observed
Hugh & Leifson (1953) test, with glucose, galactose, arabinose, xylose, fructose, sucrose, lactose and glycerol added at 1 %	14	A 0	Inert
		1	Oxidative
		2	Very slow oxidation
		3	Fermentative
			(For each of 8 sugars)
		D 1	Gas from glucose
Litmus milk	14	A 0	Not reduced
		1	Reduced
		A 0	pH unchanged
		1	Alkaline
		2	Acid
		Q –	Reduction absent
		0	Reduction partial
		1	Reduction complete
		D 1	Clot
Milk agar (Smith, Gordon & Clark, 1946)	7	A 0	Casein unchanged
		1	Casein digested
Starch agar (Smith *et al.* 1946)	7	A 0	Starch unchanged
		1	Starch hydrolysed
Loffler serum	14	A 0	Unchanged
		1	Cleared or digested
Gelatin stab	30	A 0	Unchanged
		1	Liquefaction
		Q –	Liquefaction absent
		0	Liquefaction partial
		1/d	Where d is no. of days for complete liquefaction
Blood plate (DA containing 0·5 %, v/v, horse blood)	2	A –	Blood unchanged
		0	Greening
		1	Haemolysis
H_2S from Lemco+cysteine medium (Clarke, 1953) with lead acetate paper	7	A 0	H_2S not formed
		1	H_2S formed
		Q –	H_2S not formed
		0	Degree of blackening of indicator
		1	
		2	
		3	
		4	
H_2S production in Kligler medium	14	A 0	Unchanged
		1	Blackened
Nitrate and nitrite reduction in peptone water+0·1 % KNO_3	7	A 0	NO_3 not reduced
		1	NO_3 reduced
		A –	If NO_3 not reduced
		0	NO_2 not reduced
		1	NO_2 reduced
Simmons (1926) test for citrate utilization	14	A 0	pH unchanged
		1	pH alkaline
Paton (1959) medium for gluconate utilization tested with Benedict reagent for reducing compounds	10	A 0	No growth
		1	Growth
		A –	If no growth
		0	Growth, Benedict –
		1	Growth, Benedict +

Table 3 (*contd*)

Test	Incubation period (days)	Computer coding*	Level observed
Arginine test (Thornley, 1960*a*)	14	A 0	pH unchanged or acid
		1	pH alkaline
	1, 2	A 0	Insensitive
Penicillin sensitivity (2·5 i.u. tablets) (Shewan *et al.* 1954).		1	Sensitive
		Q	Insensitive
		0	Zone < 1 mm.
Basal media: YEA for F4 strains, FA for F5		1–12	Zone size in mm. (zones > 12 mm. coded as 12)
Terramycin sensitivity (10 μg. tablets). Basal media as for penicillin	1, 2		As for penicillin
Kovacs (1956) oxidase test	—	A 0	Negative or slight trace
		1	Positive
Ability to grow at 0°. Inoculum streaked across DA plate	14	A 0	No growth, isolated colonies or slight trace
		1	Growth
Ability to grow at 37°. Inoculum streaked across DA plate. Growth checked for viability on subculture at 20°	7	A 0	No growth
		1	Slight trace, isolated colonies, or growth not viable when subcultured
		2	Growth, viable when subcultured

* D, dichotomies; A, alternatives (qualitatives); Q, quantitatives.

tube only. When such tests were repeated, the same very slight change might be confirmed, or the result might fall into the range classed as 'oxidative' or 'inert'. In the last two cases, the second result was used; but in the first case, or if the test was not repeated, it was coded as 'very slow oxidation' for the computer.

1.5.2. *Antibiotic sensitivity*

For the computer study, the sensitivity of each strain to penicillin and terramycin was tested, using 'Sentest' tablets (Shewan, Hodgkiss & Liston, 1954). Later, a selection of strains was examined by the gradient plating technique of Szybalski (1952). Benzyl penicillin (Glaxo) was added to Difco Penassay agar at concentrations of 0·5, 10 and 100 i.u./ml., and these mixtures were used to form the wedge-shaped penicillin-containing portion of each plate, to which an equal volume of the same agar without penicillin was later added. Each strain was tested on plates with these three concentrations, and some strains which failed to grow even with the lowest concentration were re-tested on plates prepared with 0·1 i.u./ml. in the penicillin-containing portion of the agar. From the results an approximate value for the minimum inhibitory concentration of penicillin for each strain was obtained.

Section 2. Computer methods

The computing was done by Mr J. C. Gower and Mr G. S. J. Ross, of the Statistics Department, Rothamsted Experimental Station, by using Classification Programme F39 for the Elliott 401 computer; this programme was later re-written and extended as CLASP for the Orion computer.

The coefficient of similarity used has been described by J. C. Gower (to be published). It allows for the inclusion of quantitative and multivalue qualitative characters as well as dichotomies, for which negative matches do not contribute to the similarity coefficient. The method of coding used for each test is listed in Tables 2 and 3.

2.1. *Choice of coding*

Tests which are scored as 'positive' or 'negative' can be coded either as dichotomies, so that negative matches are not counted, or as qualitative characters, in which case positive and negative matches contribute equally to the similarity coefficient. Sneath (1957) excluded negative matches, while Hill *et al.* (1961) and Beers & Lockhart (1962) advocated their inclusion. Since then both methods have been applied by many authors, using various similarity coefficients which were reviewed by Sokal & Sneath (1963).

The present author considers the inclusion of negative matches more satisfactory, since most tests give arbitrary results, depending on the sensitivity of reagents, conditions of incubation and similar factors. Also, either positive or negative results may have various causes; for instance, negative results could be due to lack of one or more of the enzymes concerned in the reaction, or lack of permeability to the substrate, while positive results in tests such as acid production could be due to different metabolic pathways. In the present work, therefore, positive and negative results have usually been regarded as of equal significance, and coded as qualitative characters (Tables 2 and 3). Where one test is secondary to another, however, the results have been coded so as to avoid duplication of information, and in many cases this may be conveniently done by using dichotomies. For instance, the characters motile or non-motile are coded as qualitative, and possession of polar flagella as a dichotomy, so that their absence should not contribute a second time to the similarity coefficients of pairs of non-motile strains.

For characters other than those scored as positive or negative, coding presented no problems. Only a few tests were suitable for the quantitative treatment (Table 3), and the qualitative method was obviously applicable to tests giving alternative results of equal significance; for instance, shape of organisms or colour of colonies (Table 2).

2.2. *Cluster analysis and representation of results*

After calculation of the matrix of similarity coefficients between all pairs of strains, the strains were sorted into clusters by the single linkage method (Sneath, 1957), which is described in detail by Sokal & Sneath (1963). The data obtained from this process are represented by shaded diagrams of the similarity matrix (Figs. 1, 3) and by dendrograms showing the grouping of strains at different degrees of similarity (Figs. 2, 4). The order in which clusters formed at the same similarity value are arranged after the computation is quite arbitrary, and a slight rearrangement, to bring together clusters of high mean similarity, has been incorporated in Figs. 1–4.

In order to delimit clusters which could be described and considered as possible taxonomic groups, some simplification was necessary. This was done, as described by Sokal & Sneath (1963), by choosing degrees of similarity at which well-defined clusters were separate; these were 72·5, 82·5 and 92·5% similarity in the present work. The arrangement obtained was then slightly modified on the basis of mean similarity, as explained under Results, 1.2.

The terminology of Sneath & Sokal (1962), in which these clusters or phenetic groups are referred to as 'phenons', has been used. When the term is prefixed by a number (e.g. 72·5-phenon) this shows the highest degree of similarity at which members of the phenon are grouped in the dendrogram.

2.3. *Extraction of differential characters*

From the data used in the computation it is possible to select the characters which have had most effect in separating the phenons formed. These must vary between phenons, and should be as nearly constant as possible within the phenon to be differentiated. The method of selection used was based on that of Sneath (1962, p. 312).

2.4. *Arrangement of data for computing*

The maximum number of strains which the Elliott 401 computer could deal with was 128, so the data had to be divided into two computations. As already mentioned, there were minor differences between F4 and F5 strains in the basal media used for a few tests and therefore these sets of strains were dealt with separately. In the first computation, 53 F5 isolates were compared with 75 named strains from culture collections. This gave well-defined phenons from which the representive strains were selected. The second computation contained 67 F4 strains, together with 40 strains from Computation 1, including the representative strains from each phenon.

RESULTS

Section 1. The arrangement of strains in phenons

Results of both computations are presented as shaded diagrams of the similarity matrix (Figs. 1, 3) and dendrograms, somewhat simplified by the use of triangular blocks to represent groups of strains (Figs. 2, 4).

Distinct clusters of strains, represented by triangular areas of darker shading, are visible in Figs. 1 and 3; their existence is confirmed by the arrangement shown in Figs. 2 and 4. Figure 2 shows also that most of the strains were associated at 72·5% similarity, that is, in a 72·5-phenon. This phenon contains all named strains of *Agrobacterium*, *Achromobacter* and *Alcaligenes*, as well as all the poultry 'Achromobacters' and many related groups such as *Moraxella*, *Mima* and *Bacterium anitratum*. The strains from other well-known genera, included for reference, were all outside the 72·5-phenon.

1.1. *Strains outside the 72·5-phenon*

These are listed in Table 4, and are the strains of *Pseudomonas*, *Aeromonas*, *Escherichia*, *Cellulomonas* and *Arthrobacter*. Their exclusion from the 72·5-phenon means that all similarity values to individual strains within the phenon were less than 72·5%. The mean similarity values for the whole 72·5-phenon to each of the single strains outside it were also calculated (Table 4), and showed that none of these strains was closely related to the 72·5-phenon as a whole, the highest mean similarities being 51·5% for *Escherichia coli* NCTC 9001 and 50·0% for *Arthrobacter globiforme* NCIB 8602.

Detailed relationships between strains outside the 72·5-phenon are indicated by the similarities between pairs of strains (Table 4) and by the groups shown in Fig. 2. Two *Pseudomonas aeruginosa* and three *P. fluorescens* strains (nos. 69–73) were all

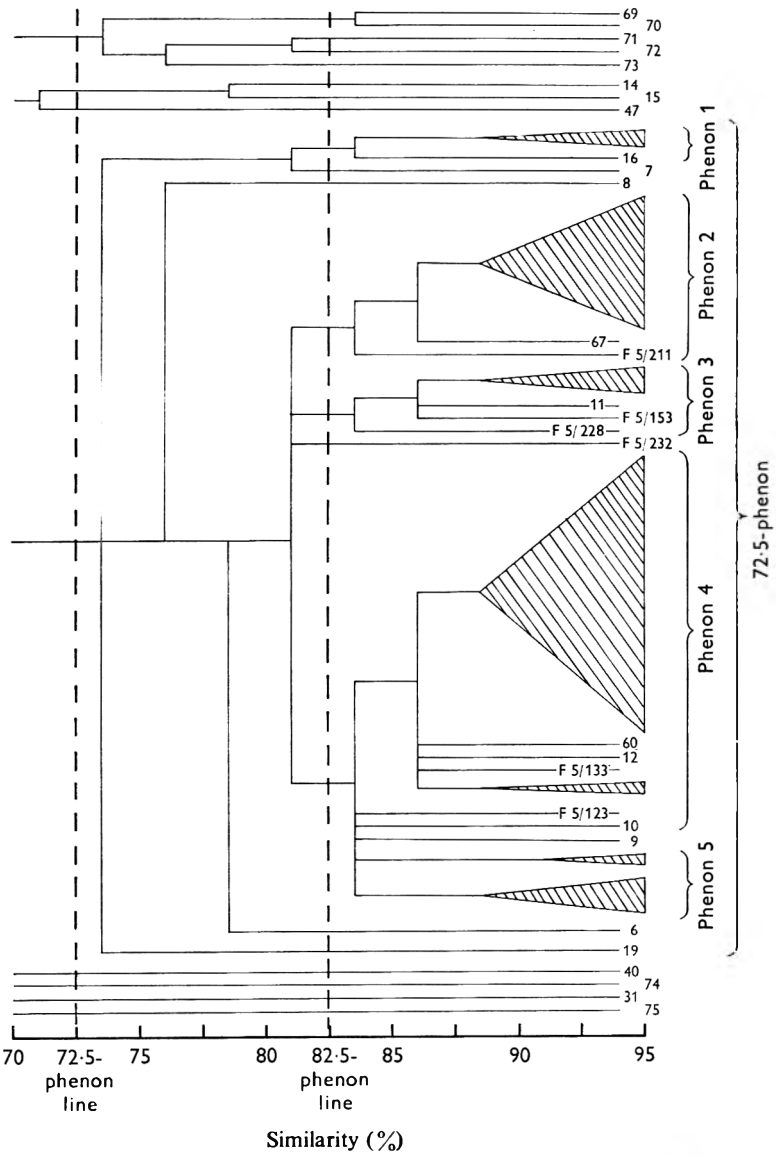

Fig. 2. Dendrogram for Computation 1. Phenons which diverge above 87·5% similarity are shown by triangles, in which the length of the base is proportional to the number of strains included. Strains are arranged in the same order as in Fig. 1.

linked together at 72·5% similarity, while the two *Aeromonas* strains (nos. 14, 15) were grouped together at 77·5% and linked with *Escherichia coli* (no. 47) at 70% similarity. Other strains outside the 72·5-phenon showed no relationships at or above 70% similarity. *Pseudomonas saccharophila* NCIB 8570 and *P. putrefaciens* NCIB 8615 (nos. 75, 74) had low similarities to the strains of *P. aeruginosa* and *P. fluorescens* (Table 4).

Table 4. *Relationships of strains outside the 72·5-phenon: mean similarity of each strain to the whole 72·5-phenon and similarity coefficients between pairs of strains*

Strain no.	Group or strain	Mean similarity values	Similarity coefficients between pairs of strains											
—	72·5-phenon	65·5*	—	—	—	—	—	—	—	—	—	—	—	—
69	*Pseudomonas aeruginosa* NCTC 6750	42·0	—	—	—	—	—	—	—	—	—	—	—	—
70	*P. aeruginosa* NCTC 2000	41·0	83·0	—	—	—	—	—	—	—	—	—	—	—
71	*P. fluorescens* NCTC 4755	44·5	60·5	74·5	—	—	—	—	—	—	—	—	—	—
72	*P. fluorescens* NCIB 3756	45·0	54·5	73·5	82·5	—	—	—	—	—	—	—	—	—
73	*P. fluorescens* NCTC 10,038	35·0	66·5	75·0	77·0	76·5	—	—	—	—	—	—	—	—
75	*P. saccharophila* NCIB 8570	45·5	57·0	66·5	59·0	54·5	56·0	—	—	—	—	—	—	—
74	*P. putrefaciens* NCIB 8615	39·5	53·0	50·0	31·5	33·5	40·5	56·5	—	—	—	—	—	—
14	*Aeromonas formicans* 18	34·5	53·0	63·0	50·0	44·0	51·0	54·5	55·5	—	—	—	—	—
15	*A. liquefaciens* NCTC 7812	29·5	51·0	54·5	52·0	48·0	53·5	43·0	48·0	79·5	—	—	—	—
47	*Escherichia coli* NCTC 9001	51·5	33·5	43·5	47·0	37·5	31·5	48·5	42·5	71·5	60·5	—	—	—
40	*Cellulomonas biazotea* NCIB 8077	37·0	29·0	31·5	42·0	38·5	26·0	51·5	39·5	64·5	54·0	68·0	—	—
31	*Arthrobacter globiforme* NCIB 8602	50·0	51·5	44·0	40·5	34·5	25·0	50·0	45·0	32·0	28·5	46·0	46·5	—
		72·5-phenon	69	70	71	72	73	75	74	14	15	47	40	31

Strain no.

* Mean similarity within the phenon.

1.2. *Subdivision of the 72·5-phenon*

In Computation 1 the 72·5-phenon contained 116 strains. Consideration of the dendrogram (Fig. 2) shows that the small group marked Phenon 1 was separate from the rest of the 72·5-phenon at 75% similarity, while nearly all other strains remained associated at 80% similarity. At 82·5% similarity four phenons were separate, the last being very large and subdivided as Phenons 4 and 5 in Fig. 2. Phenons 1–3 in Fig. 2 appeared to be fairly homogeneous distinct clusters in Fig. 1, and gave mean similarities within each phenon of 83–85% (Table 5). When mean similarities of each strain to all other strains in the same phenon were calculated (Table 6), values for strains in Phenons 1–3 were mostly above 80%, and none was lower than 75%.

Table 5. *Mean similarities within phenons and between phenons*

Compn.	No. of strains	Phenon	Mean similarities				
1	4	1	85·0	—	—	—	—
	28	2	57·0	84·0	—	—	—
	8	3	60·5	69·5	83·0	—	—
	61	4	40·5	56·5	63·5	79·0	—
	9	5	52·5	55·0	51·5	64·5	83·0
		Phenon:	1	2	3	4	5
2*	9	2	84·0	—	—	—	—
	18	3	67·5	88·0	—	—	—
	64	4	56·5	69·5	80·5	—	—
	3	5	54·0	53·0	65·0	87·5	—
		Phenon:	2	3	4	5	—

* In Computation 2, Phenon 1 contained only 2 strains, and has been omitted

The fourth 82·5-phenon, containing 71 strains, MJT/F5/12A to 26 (Fig. 1), was clearly not homogeneous, and the portion marked Phenon 5 seemed particularly distinct from the rest. The mean similarity for the whole 82·5-phenon (strains MJT/F5/12A to 26) was 76·0%, and mean similarities for each strain compared with the rest of the phenon ranged from 61·5 to 82·0%. Eight strains had mean similarities below 70%, and, of these, seven were in the portion marked Phenon 5. Mean similarities were therefore found separately for the portions marked Phenons 4 and 5 on Figs. 1 and 2, and gave within-phenon means of 79·0 and 83·0% respectively (Table 5). As the mean similarity between Phenons 4 and 5 was only 64·5%, while that between Phenons 2 and 3 was 69·5% (Table 5), the separation of Phenons 4 and 5 was considered desirable. This appears to be a case where the relationship revealed by the single-linkage method of clustering is not the same as that based on mean similarities, probably because of the existence of intermediate strains, such as strain 9 (*Achromobacter* A16). The clustering based on mean similarities was preferred, and Phenons 4 and 5 were considered separately.

The boundaries of Phenons 4 and 5 were therefore also based on mean similarity, which caused the inclusion in Phenon 4 of strains MJT/F5/123 and 10 (see Table 6) and the exclusion of strain 9, which was equally related to Phenons 4 and 5 (Table 8). In Phenon 5 two separate branches of the dendrogram, one of which contained only two strains, were considered together for similar reasons.

Table 6. *Strains within Phenons 1–5, arranged in order of highest mean similarity to the other strains in the same phenon*

Phenon	Mean S†	Strain no. and designation
1	87·5	18* *Agrobacterium* ZH 1
	85·0	2* *Achromobacter hartlebii* NCIB 8129
	84·5	17 *Agrobacterium* LK 10
	83·5	16 *A. tumefaciens* NCIB 8150
2	88·5	39* *Bacterium anitratum* B 25
	88·0	3* *Achromobacter lacticum* NCIB 8208
	87·5	48* *Herellea* Z 6
	87·5	50* *Herellea* Z 8
	87·0	38 *Bacterium anitratum* B 24
	87·0	37 *B. anitratum* B 16
	87·0	36 *B. anitratum* B 11
	86·5	41* *Diplococcus mucosus* 169
	86·0	4 *Achromobacter lacticum* NCIB 8209
	86·0	45 *Diplococcus mucosus* E 2241/60
	85·0	55* *Herellea* 6009
	85·0	46 *Diplococcus mucosus* E 8743/60
	84·5	35 *Bacterium anitratum* B 10
	84·5	51 *Herellea* 5937
	84·5	44 *Diplococcus mucosus* SO 1506/61
	84·0	13 *Acinetobacter anitratum* NCIB 9019
	84·0	43 *Diplococcus mucosus* SO 1472/61
	83·5	49 *Herellea* Z 7
	83·5	33 *Bacterium anitratum* B 7
	82·5	54 *Herellea* 5944
	81·5	52 *Herellea* 5939
	81·0	34* *Bacterium anitratum* B 9
	80·5	42* *Diplococcus mucosus* 019
	80·5	1* *Achromobacter anitratus* NCTC 8102
	80·5	53 *Herellea* 5942
	79·0	32 *Bacterium anitratum* B 5
	76·0	67* *Moraxella lwoffi* 148/57
	75·0	MJT/F5/211
3	86·5	MJT/F5/158*
(Compn. 1)	84·0	MJT/F5/154*
	84·0	MJT/F5/153*
	83·5	4 F5 isolates
	80·0	11* *Achromobacter* 138
3	92·0	MJT/F4/6/20
(Compn. 2)	92·0	MJT/F4/6/5
	90·5	MJT/F4/6/17 B
	90·5	MJT/F4/6/11
	90·0	MJT/F4/153*
	90·0–85·0	10 F4 isolates
	85·0	MJT/F5/154*
	84·0	MJT/F4/3/4
	83·5	MJT/F5/158*
4	84·5	MJT/F5/111*
(Compn. 1)	84·5	MJT/F5/122
	84·5	MJT/F5/212*
	84·5	MJT/F5/120
	84·0	MJT/F5/284*
	84·0–83·0	5 F5 isolates
	82·5	MJT/F5/199A
	82·5	68* *Neisseria catarrhalis* NCTC 3622
	82·5–81·0	7 F5 isolates
	81·0	MJT/F5/238

Table 6 (*contd*)

Phenon	Mean S†	Strain no. and designation
4 (Compn. 1) (*cont.*)	81·0	MJT/F5/56*
	81·0	59* *Mima* Z 4
	80·5	MJT/F5/239*
	80·5	MJT/F5/136
	80·0	MJT/F5/14
	80·0–79·5	8 F5 isolates
	79·5	5* *Achromobacter venenosum* NCIB 9022
	79·0	3 F5 isolates
	79·0	62* *Mima* 5979
	78·5	56 *Mima* Z 1
	78·5	MJT/F5/236
	78·0	28* *Alcaligenes viscosus* NCIB 8596
	77·5	2 F5 isolates
	77·0	57 *Mima* Z 2
	76·5	61 *Mima* 5936
	76·5	MJT/F5/76
	76·0	66* *Moraxella lwoffi* 950/56
	75·5	2 F5 isolates
	75·5	27* *Alcaligenes viscosus* NCIB 8154
	75·0	64 *Moraxella lwoffi* NCTC 5867
	75·0	MJT/F5/126
	74·5	58 *Mima* Z 3
	74·0	MJT/F5/123
	74·0	MJT/F5/133
	74·0	65 *Moraxella lwoffi* NCTC 7976
	74·0	12* *Achromobacter* 25A 2
	73·5	63* *Moraxella lwoffi* NCTC 5866
	73·0	60 *Mima* 5902
	73·0	10* *Achromobacter* 131
	72·5	MJT/F5/12 A
4 (Compn. 2)	86·0	MJT/F4/8/4B
	85·5	MJT/F5/56*
	85·5	MJT/F4/18/27
	85·5	MJT/F4/6/7
	85·0–84·5	7 F4 isolates
	84·5	MJT/F5/239*
	84·0	MJT/F5/284*
	84·0–83·5	2 F4 isolates
	83·5	59* *Mima* Z 4
	83·0	MJT/F5/111*
	83·0	MJT/F4/5/7
	83·0	2 F4 isolates
	82·5	MJT/F4/8/3
	82·5–82·0	4 F4 isolates
	82·0	MJT/F5/212*
	81·5	7 F4 isolates
	81·0	68* *Neisseria catarrhalis* NCTC 3622
	81·0	3 F4 isolates
	80·5	MJT/F4/21/9
	80·5	62* *Mima* 5979
	80·5–76·5	16 F4 isolates
	76·5	66* *Moraxella lwoffi* 950/56
	76·5	MJT/F4/2/6 A
	76·0	28* *Alcaligenes viscosus* NCIB 8596
	75·5	63* *Moraxella lwoffi* NCTC 5856
	75·0	5* *Achromobacter venenosum* NCIB 9022
	75·0–73·0	2 F4 isolates
	71·0	27* *Alcaligenes viscosus* NCIB 8154
	71·0	MJT/F4/3/16

Table 6 (*contd*)

Phenon	Mean S†	Strain no. and designation
5	86·5	20* *A. faecalis* NCTC 8764
	86·0	29* *Alcaligenes* CS 8
	85·0	26 *A. viscosus* NCTC 3233
	83·5	30 *Alcaligenes* CS 11
	82·0	21 *A. faecalis* NCTC 655
	82·0	25 *A. denitrificans* NCTC 8582
	81·0	24* *A. bookeri* NCIB 8155
	80·5	23* *Alcaligenes* sp.
	78·0	22 *A. faecalis* NCTC 8769

* Indicates strains included for reference in both computations.
† Mean similarity of the strain indicated to the other strains in the same phenon.

Strains having the highest mean similarity to other strains in the same phenon (Table 6) were regarded as representative strains.

When selecting strains for Computation 2, strains outside the 72·5-phenon of Computation 1 were not included. Representative strains from each of the five smaller phenons, together with other named strains of particular interest from Computation 1, were compared with 67 additional poultry isolates, from the F4 experiment.

The picture obtained (Figs. 3, 4) was in general similar to that from Computation 1, with well-defined clusters which could be identified with the phenons of Computation 1 by the presence of the representative strains. In both computations almost all the poultry isolates appeared in Phenons 3 and 4, and, as the only additional material in Computation 2 was the collection of poultry isolates from Expt. F4, it follows that only the relationships of Phenons 3 and 4 might be expected to differ appreciably. In fact, these two phenons were rather more closely related in Computation 2, both on the dendrogram, where they were linked at 85% similarity (Fig. 4) compared with 80% similarity in Fig. 2, and on the basis of mean similarity between the two phenons, which was 69·5% compared with 63·5% in the earlier computation (Table 5). Relationships with the other phenons were, however, much the same as in the first computation (Table 5).

In the detailed results which follow, descriptions of Phenons 1, 2 and 5 are based on the data from Computation 1, since these phenons consisted almost entirely of named strains, and were more fully represented in this computation. For Phenons 3 and 4, containing a large proportion of poultry isolates, data from the two computations have been combined. Table 6 lists the strains included in each phenon, in decreasing order of mean similarity to the other strains in the phenon.

1.3. *Phenon 1*

Representative strain: 18 *Agrobacterium* ZH 1. This phenon contained the three strains of *Agrobacterium* included in the survey, and *Achromobacter hartlebii* NCIB 8129. The soil *Achromobacter* CB 11 (strain 7) was also close to this group but was not included within the limits of 82·5% similarity.

1.4. *Phenon 2*

Representative strain: 39 *Bacterium anitratum* B 25 (B5W99 of Ferguson & Roberts, 1950). This phenon consisted of 28 strains, mostly of human origin. It included all

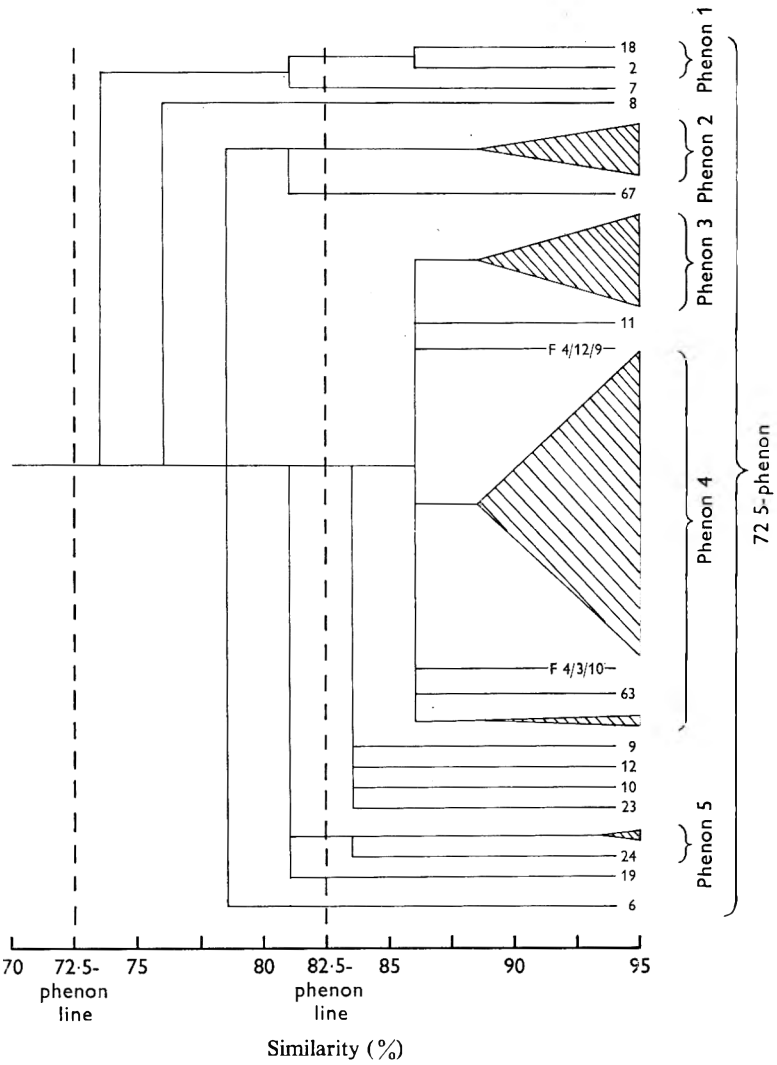

Fig. 4. Dendrogram for Computation 2. Triangles are used as in Fig. 2.

strains named *Bacterium* (or *Achromobacter*, or *Acinetobacter anitratum* (10 strains), *Achromobacter lacticum* (2 strains), *Herellea* (8 strains), *Diplococcus mucosus* (6 strains), one glucose-oxidizing form of *Moraxella lwoffi* and one glucose-oxidizing poultry isolate. The very close relation between strains named *Bacterium anitratum*, *Achromobacter lacticum*, *Herellea* and *Diplococcus mucosus* is shown by the fact that representatives of all these appear near the top of the list in Table 6, with mean similarity values above 86%.

1.5. *Phenon 3*

Representative strains: MJT/F5/158, MJT/F4/6/20. In Computation 1 this contained only 8 strains, of which 7 were isolated from poultry in Expt. F5 and one was from

fish. The three with highest mean similarities were included in Computation 2, where they were grouped with 15 additional strains, isolated from poultry in Expt. F4.

However, the strain with the highest mean similarity in Computation 1 (MJT/F5/158) had the lowest mean similarity in Computation 2, which indicates slight differences in properties of F4 and F5 strains belonging to this phenon.

Table 7. *Strains within Phenons 4i, 4ii and 4iii, arranged in order of highest mean similarity to the other strains in the same phenon*

Phenon	Mean S†	Strain no. and designation
4i	94·5	MJT/F5/238
(Compn. 1)	94·0	MJT/F5/239*
	93·5–92·0	2 F5 isolates
	92·0	MJT/F5/56*
	91·0–89·5	4 F5 isolates
	89·0	59* *Mima* Z 4
4ii	94·5	MJT/F5/122
(Compn. 1)	94·5	MJT/F5/212*
	94·5–92·5	7 F5 isolates
	92·5	MJT/F5/111*
	92·0–90·0	6 F5 isolates
	90·0	MJT/F5/284*
	89·0	1 F5 isolate
4iii	90·5	MJT/F5/14
(Compn. 1)	90·0	MJT/F5/5
	89·5	61 *Mima* 5936
	89·5	56 *Mima* Z 1
	87·5	57 *Mima* Z 2
	87·5	MJT/F5/15
	86·0	MJT/F5/96
4i	94·0	MJT/F5/239*
(Compn. 2)	94·0	MJT/F4/6/14
	94·0–93·0	7 F4 isolates
	92·5	MJT/F5/56*
	92·0–88·5	8 F4 isolates
	88·5	59* *Mima* Z 4
	88·0–86·0	7 F4 isolates
4ii	93·0	MJT/F4/5/7
(Compn. 2)	92·0	MJT/F5/284*
	92·0–89·0	8 F4 isolates
	89·0	MJT/F5/212*
	87·5–86·5	4 F4 isolates
	85·5	MJT/F5/111*
	83·5	1 F4 isolate

* Indicates strains included for reference in both computation.
† Mean similarity of the strain indicated to the other strains in the same phenon.

In the earlier work (Thornley, 1960*b*) seven strains of 'Achromobacter' were placed in Group A, and, of these strains, six were included in Phenon 3 and one (MJT/F5/232) was close to it (see Table 8). It is evident that Phenon 3 corresponds to Group A of the earlier study.

1.6. *Phenon 4*

Representative strain: MJT/F5/111. In Computation 1 this phenon contained 61 strains, made up of 44 poultry isolates from Expt. F5, *Mima* (seven strains), *Moraxella*

lwoffi (four strains which did not produce acid from sugars), *Achromobacter venenosum* NCIB 9022, *Achromobacter* strains 131 and 25A2, and two strains of *Alcaligenes viscosus*. *Neisseria catarrhalis* NCTC 3622 was also included, but not so much information was available for comparison here, as the strain did not grow in six of the tests used.

Five poultry strains with high mean similarities, together with several named strains, were included in Computation 2, where they were grouped with 51 isolates from Expt. F4.

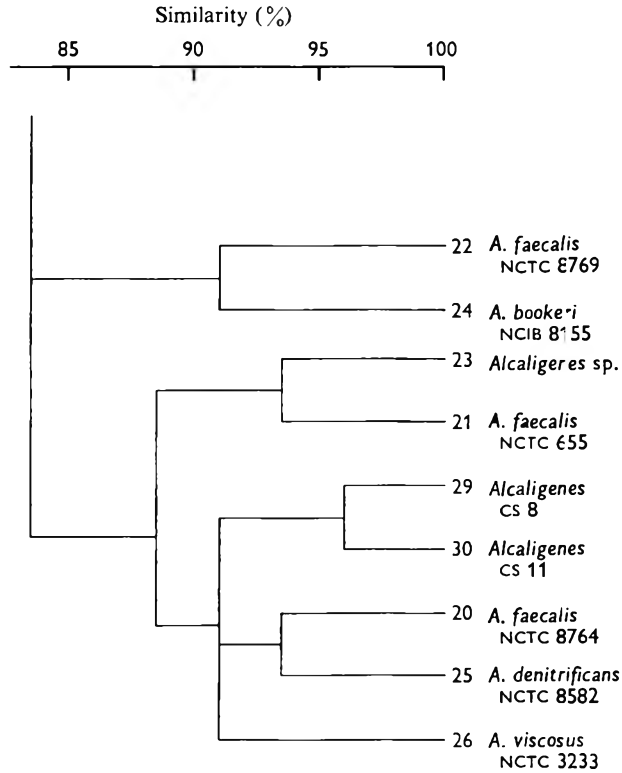

Fig. 5. Detailed dendrogram of Phenon 5, Computation 1, shown by two triangles in Fig. 2.

Several smaller clusters of strains, with very high similarities, were present within Phenon 4, and are indicated in Figs. 1 and 3. In Computation 1, three of these small phenons were apparent: 4i, 4ii, 4iii. Representative strains from Phenons 4i and 4ii were included in the second computation, and were present in the phenons labelled 4i and 4ii in Fig. 3. Phenon 4iii did not appear in Computation 2. Three of these small phenons were 92·5-phenons, while the other two (4i, 4ii, Compn. 1) were based on 92·5-phenons, but the limits were slightly altered on the basis of mean similarity. Many strains in Phenon 4 were not grouped into any of these small subgroups.

The strains included in Phenons 4i–4iii are listed in Table 7, and include poultry isolates, and four strains of *Mima*, one of which appeared in 4i, and three in 4iii. Representative strains are: Phenon 4i: MJT/F5/239; Phenon 4ii: MJT/F5/122, MJT/

F4/5/7; Phenon 4iii: MJT/F5/14. When the grouping is compared with that of Thornley (1960*b*) for 25 isolates from poultry, the same three strains are placed in Group C and Phenon 4iii, which therefore correspond, while the six strains of Group B are divided between 4i (3 strains), 4ii (1 strain) and the ungrouped part of Phenon 4 (2 strains).

Among the named strains in Phenon 4, three strains of *Mima* (56, 57, 61) were grouped in Phenon 4iii, and three strains of *Moraxella lwoffi* (63, 64, 65) formed a small 92·5-phenon by themselves (Fig. 1). The two strains of *Alcaligenes viscosus* (27, 28) were linked together at 87·5% similarity, and with the rest of Phenon 4 at 85% (Fig. 2). However, their mean similarity values to the rest of the phenon, 78% and 75·5% (Table 6), show there is no doubt that they should be included in Phenon 4. All other named strains in Phenon 4 were scattered singly among the poultry isolates.

Table 8. *Strains included in the 72·5-phenon but not in Phenons 1–5, and their relationships based on mean similarity to phenons*

Strain no. and designation	Phenon with highest mean similarity to the strain in: Computation 1	Computation 2
7. *Achromobacter* CB 11	1	1
8. *Achromobacter* 7A 14	1 and 5	1 and 5
MJT/F5/232	3	–*
MJT/F4/12/9	—*	3
9. *Achromobacter* A 16	4 and 5	4
6. *Achromobacter* EB/F64/100	4 and 5	4 and 5
19. *Alcaligenes faecalis* NCTC 415	5	5

* Strain not studied in this computation.

1.7. *Phenon 5*

Representative strain: 20 *Alcaligenes faecalis* NCTC 8764. Apart from two strains of *Alcaligenes viscosus*, in Phenon 4, all strains named *Alcaligenes* were placed in Phenon 5, including three strains received as *A. faecalis*, the type species. Their arrangement according to mean similarity is shown in Table 6, and the dendrogram (Figs. 2, 5) shows that two small phenons separate at 85% similarity were included, the first consisting of two strains (22, 24) only, and the second being an 87·5-phenon containing seven strains.

Strain 23 was thought, when the testing and computation were done, to be NCIB 8156 (*A. faecalis*); a culture of this strain obtained later gave different results, but which agreed with those of Hendrie, Hodgkiss & Shewan (1964). As strain 23 had not been kept, it was impossible to re-test it and its properties have been omitted from Section 2.

1.8. *Strains within the 72·5-phenon but not included in Phenons 1–5*

The closest affinities of these strains are shown in Table 8, and their properties are given in Section 2.

Section 2. The properties of phenons and individual strains

All the strains included in this survey were Gram-negative or Gram-variable, did not form spores, were able to grow on HIA or YEA media, and none was an obligate anaerobe.

2.1. *Strains outside the 72·5-phenon*

Table 9 shows that the characters which were uniform or nearly so throughout the very large 72·5-phenon were all negative. The twelve strains outside this phenon were distinguished by various combinations of positive characters, such as the fermentation of sugars in Hugh & Leifson medium, pigmentation, production of alkalinity from arginine, and proteolytic properties.

Table 9. *Characters which differentiate the 72·5-phenon from strains outside it*

Character	72·5-phenon (183 strains)	Strains outside the 72·5-phenon											
		P. aeruginosa		*P. fluorescens*			*Aeromonas*		*E. coli*	*Cellulomonas*	*P. putrefaciens*	*Arthrobacter*	*P. saccharophila*
		69	70	71	72	73	14	15	47	40	74	31	75
Gas from glucose	−	.	.	.	.	.	.	+	+	.	.	.	.
Fermentation of:													
Glucose	−	.	.	.	.	.	+	+	+	+	.	.	.
Galactose	−	.	.	.	.	.	+	+	+	+	.	.	.
Xylose	−	.	.	.	.	.	.	.	.	+	.	.	.
Arabinose	−	.	.	.	.	.	+	+	+	+	.	.	.
Lactose	−	.	.	.	.	.	+	.	+	+	.	.	.
Sucrose	−	.	.	.	.	.	+	+	.	+	.	.	.
Fructose	−	.	.	.	.	.	+	+	+	+	.	.	.
Glycerol	−	.	.	.	.	.	+	+	+	+	.	.	.
Fluorescent pigment	−	+	+	+	+	+	.	.	.	.	.	.	.
Colony colour:													
Yellow	−	.	.	.	.	.	.	.	.	+	.	.	+
Brown	−	.	.	.	.	.	.	.	.	.	+	.	.
Reducing compounds from gluconate	−	+	+	.	+	+	.	+	.	.	.	.	.
Arginine	− (98 %)	+	+	+	+	+	+	+	.	.	.	.	.
Digestion of serum	− (98 %)	+	+	.	.	+	+	+	.	.	+	.	.
Casein hydrolysis	− (98 %)	+	+	.	+	+	+	+	.	.	+	.	.
Starch hydrolysis	− (99 %)	.	.	.	.	.	+	+	.	+	.	.	.
Gelatine liquefaction	− (94 %)	+	+	+	+	+	+	+	.	+	−	+	+

2.2. *Strains within the 72·5-phenon*

2.2.1. *The differentiation of Phenons 1–5 and their subdivisions*

Of the characters which varied within the 72·5-phenon, those which had been most effective in separation of Phenons 1–5 were selected (Methods, 2.3) and are listed in Table 10. Characters of no use for differentiation are omitted. The highest discriminatory value was shown by tests which gave different but uniform results in different phenons; only three tests (oxidative acid production from sucrose, fructose and xylose) did this. The discriminatory value of other tests can be judged by the nearness to 100% of the results for each phenon in Table 10. Such data are useful as a first step in the selection of tests for identification; other criteria which are important for this purpose, such as reproducibility of the tests, were not studied here.

Table 10. *Differential characters for Phenons 1 to 5*

Phenon		1	2	3	4	5
Number of strains		4	28	23	110	8
Morphology of cells		r	cr (75)*, sr (12), c (13)	cr (91), sr (9)	cr (88), sr (6), c (6)	r (88), sr (12)
Motility		+(75)	−	−	−	+(75)
Colonies		tr	op (86)	op (87)	op (70)	tr (75)
Oxidative acid production† from:	glycerol	+(75)§	−			−
	sucrose	+	−	−	−	−
	fructose	+	−	−	−	−
	lactose	+	+(96)	+(96)§	−	−
	arabinose	+	+	+(96)§	−	−
	xylose	+	+	+	−	−
	galactose	+	+	+(96)§	−	−
	glucose	+	+	+(96)§	−	−
H_2S (Kligler)		+	−	−	−(92)	−
Oxidase		+	−	+(96)	−(55)	+
NO_3 reduction		+(75)	−(89)	+(91)	−(55)	+(88)
Citrate		+	+(93)	−(78)	−(84)	+
Gluconate		+	+(68)	−(74)	−(51)	−
Growth at 0°		−	−(75)	+	+(83)	−(88)
Growth at 37°		−(75)	+(96)	−(87)	−(80)	+
Litmus milk (pH change)		alk (50), ac (25)	ac (79)	uc (74), ac (26)	uc (88)	alk (75)
Penicillin sensitivity (2·5 i.u. tablets)		−	−(93)	+	+(79)	−

Abbreviations: r = rod, sr = short rod, cr = coccoid rod, c = coccus, tr = translucent, op = opaque, alk = alkaline, ac = acid, uc = unchanged.
* Percentage of strains giving the indicated results
† Results called 'very slow oxidation' or 'inert' are both listed as negative.
§ Negatives all due to 'very slow oxidation'.

2.2.1.1. *Morphology*

The morphological properties useful in differentiation of phenons can be seen from Table 10. Additional information, mainly based on electron microscopy, is included in this section.

Phenon 1. The four strains forming this phenon were small rods, of which only three strains were motile in broth (Table 10); the exception, *Agrobacterium* NCIB 8150, was motile in Hugh & Leifson medium.

In the electron microscope one or two lateral flagella per organism were seen on *Agrobacterium* LK 10. *Agrobacterium* ZH 1 was shown to be peritrichous in the electron micrographs of Hendrie *et al.* (1964), who also reported *Achromobacter hartlebii* NCIB 8129 to be peritrichous; but in the author's flagella stains some organisms had a few lateral flagella while others appeared to have a single polar flagellum. This observation has not been confirmed by electron microscopy, however.

Colonies of all four strains were very small, cream coloured and translucent after incubation for 3 days at 25° on HIA, and were thus slower growing than almost all others in the survey.

Phenons 2, 3 and 4. Strains in these three phenons were very similar in morphology, and they will be described together. Table 10 shows that all these strains were non-motile and consisted of coccoid rods, cocci or short rods.

Most strains were Gram-negative, but often appeared a darker red than is usual for Gram-negative bacteria, possibly due to retention of some of the crystal violet. Some of the author's strains from poultry, especially when freshly isolated, showed a proportion of Gram-positive organisms in smears. These strains are referred to as 'Gram-variable', and they formed 13% of the strains in Phenon 3 and 14% of strains in Phenon 4.

In shape, the organisms of most strains were classed as coccoid rods by light microscopy (Table 10). Electron microscopy of negatively-stained preparations showed grooves (Pl. 1, fig. 1, *G*) where division was taking place, and some organisms were therefore more accurately described as cocci than as coccoid rods. This difference between light microscope and electron microscope observations has been mentioned for similar bacteria by Lemoigne, Girard & Jacobelli (1952). Strains classed as coccoid rods by light microscopy and cocci by electron microscopy are illustrated in Pl. 1, figs. 1, 2 and 3, while Pl. 2, fig. 4, shows a strain consisting mainly of coccoid rods, as judged by both methods.

The various named strains which were grouped in Phenon 2 (Table 6) were mostly classed as coccoid rods (Table 10), and this agreed with Klinge's (1959) electron micrographs of strains named *Bacterium anitratum*, *Diplococcus mucosus* and *Moraxella lwoffi*. In both Klinge's work and the present study strains previously named 'Diplococcus' were not more coccoid than the others studied.

Named strains in Phenon 4 included four strains of *Moraxella lwoffi*, which were short rods; seven strains received as *Mima*, of which two were cocci and four coccoid rods (by light microscopy); and one strain of *Neisseria catarrhalis*, which consisted of cocci.

In electron micrographs fimbriae were visible on many strains (Pl. 1, figs. 1, 2, Pl. 2, fig. 4, *F*) and also capsular material which appeared dark because of its retention of phosphotungstate (Pl. 1, figs. 1, 2, *C*).

In many strains, organisms were grouped in pairs (Pl. 1, fig. 2) and small clumps (Pl. 1, fig. 1); short chains and tetrads were also observed. The two strains of *Alcaligenes viscosus*, in Phenon 4, showed a tendency to form long chains, which were cocci in NCIB 8596 (Pl. 1, fig. 3) and coccoid rods (by electron microscopy) in NCIB 8154. It is not known whether the material connecting the organisms in the chain was mucilaginous, or part of the cell walls.

The grouping of organisms in either short chains or tetrads, due to the direction of successive planes of division being either parallel or perpendicular, has been considered important in classification, the former method being thought characteristic of *Moraxella* and the latter of *Neisseria* (Murray & Truant, 1954; Piéchaud, 1961). One poultry strain (MJT/F4/199A) was studied in detail by electron microscopy (Thornley & Glauert, to be published) and thin sections showed that both methods of division occurred at different stages of the culture. Plate 2, fig. 5 shows a section through a short chain of organisms in a 10 hr culture in HIB medium grown with aeration at 25°. An older septum in the centre of the chain divides two pairs of organisms each separated by a more recently formed septum. These septa are all parallel, and this method of division was predominant in this culture. Plate 2, fig. 6 shows the same strain after growth in the same conditions for 24 hr when all groups containing four organisms were in the form of tetrads. Two organisms are united by a recently formed septum. One of them shows a narrow connecting-link to a third organism of the tetrad; this remains from an earlier division at right angles to the latest one.

The colonies produced by strains in Phenons 2, 3 and 4 were generally more opaque than those of Phenons 1 and 5 (Table 10). The term 'opaque' was relative, and was used when comparing colonies of Gram-negative strains; it was surprising to find a correlation between the predominantly opaque colonies and the coccoid rod morphology, which existed in Phenons 2, 3 and 4. The colonies were cream, and in most strains were smooth, entire and convex, with a butyrous consistency. Some poultry isolates in Phenons 3 and 4 produced colonies with a more gluey consistency, which pulled out into strands when picked with an inoculating needle. An even more coherent form was found in a few poultry isolates of Phenon 4; this latter colony appeared dull and wrinkled, and could only be subcultured by removing the whole colony from the agar plate. One strain in Phenon 4 produced a deep brown diffusible pigment, but was similar to the rest of the phenon in other properties.

Phenon 5. Most strains consisted of rod-shaped organisms, while a few were short rods (Table 10), the shortest being strain 21, *Alcaligenes faecalis* NCTC 655 (Pl. 2, fig. 7). Strain 26 (*A. viscosus* NCTC 3233) consisted of slender tapered rods, some slightly curved; no other organism studied had the same microscopic appearance. Motility in broth cultures was only observed with six of the eight strains (Table 10), but the other two strains (21, 26) appeared motile in Hugh & Leifson medium, and strain 21 showed a few lateral flagella (Pl. 2, fig. 7), which confirmed the description of this strain as peritrichous by Hendrie *et al.* (1964). Fimbriae were also present in this strain (Pl. 2, fig. 7). The flagellation of strain 26 was not determined. Peritrichous flagella were observed for strain 20 (*A. faecalis* NCTC 8764), 29 (*Alcaligenes* CS 8) and 30 (*Alcaligenes* CS 11), as stated by Hendrie *et al.* (1964), and also for strain 25 (*A. denitrificans* NCTC 8582). The two strains grouped in a small phenon, separate at 85% similarity from the rest of Phenon 5 (Results, 1.7), both had polar flagella. These

were strain 22 (*A. faecalis* NCTC 8769), previously described as polarly flagellate by Hendrie *et al.* (1964), and strain 24 (*A. bookeri* NCIB 8155).

Colonies were usually cream, translucent, smooth and entire, while some were rough, convex in the centre with a thin spreading irregular edge.

2.2.1.2. *Biochemical properties.* These will not be described for each phenon separately since the differential properties for Phenons 1–5 are shown in Table 10. As explained in the Discussion, Phenons 2, 3 and 4 are considered to belong to *Acinetobacter*, and for convenience all the data on these phenons and their subdivisions are collected in Table 11; the differential properties for the subdivisions of Phenon 4 are marked D in this table.

The Hugh & Leifson method of studying sugar metabolism differentiated the following groups of phenons: Phenons 4 and 5, containing strains which produced no acid from the eight compounds studied; Phenons 2 and 3, which showed oxidative acid production from five sugars (glucose, galactose, arabinose, xylose and lactose) and Phenon 1, in which most strains produced acid oxidatively from all eight compounds. Production of H_2S in Kligler medium was another characteristic of Phenon 1 which was absent from nearly all strains in other phenons.

A positive result in Kovacs (1956) oxidase test and the ability to reduce nitrate seemed to be correlated among strains within the 72·5-phenon, and positive results were useful in differentiating Phenons 1, 3 and 5 from Phenon 2, in which predominantly negative results were found. Strains within Phenon 4 varied in results, as the two tests differentiated the subdivisions of Phenon 4 (Table 11).

Ability to grow with citrate as sole carbon source and an inorganic nitrogen source was present in most strains of Phenons 1, 2 and 5, while generally absent in Phenons 3 and 4 (Table 10). Growth with gluconate as sole carbon source was also found in Phenon 1, but was less common in Phenons 2 and 5 than was growth with citrate. In Phenons 4ii and 4iii, however, most strains could grow with gluconate although not with citrate as sole carbon source (Table 11). The majority of strains in Phenons 3 and 4i failed to grow in either test, and this may have been due to a requirement for organic nitrogen or other growth factors rather than an effect of the carbon source.

Other differential tests for Phenons 1–5 were growth temperature, change of pH in litmus milk and penicillin sensitivity (see Results, 2.4).

Subdivisions of Phenon 4. Differential tests for Phenons 4i–4iii within Phenon 4 included some of those already mentioned (oxidase, nitrate, gluconate, pH change in litmus milk and penicillin sensitivity) and, in addition, reduction of litmus milk, production of H_2S from cysteine and sensitivity to terramycin.

Nearly all the named strains of *Moraxella* and *Mima* included in Phenon 4 differed from the majority of strains in growth temperatures, since they were able to grow at 37° but not at 0°. In other properties, *Mima* z 4 resembled the rest of Phenon 4i, except that it was unable to reduce nitrate, three strains of *Mima* (56, 57, 61) had properties typical of Phenon 4iii, and three strains of *Moraxella lwoffi* (63–65) formed a separate 92·5-phenon with properties listed in Table 11. Other named strains of human origin were scattered singly among the poultry isolates in the ungrouped portion of Phenon 4, indicating that they had different combinations of properties from the subdivisions of the phenons and from each other.

Subdivisions of Phenon 5. The two small phenons within Phenon 5 (Fig. 5) differed

Table 11. *List of all characters of strains in Phenons 2, 3 and 4; these are classified as Acinetobacter and Phenon 2 represents A. anitratus*

1 Characters common to all strains: Gram-negative or variable, non-spore-forming, non-motile coccoid rods cocci or short rods, often in pairs; able to grow aerobically on nutrient media such as HIA or YEA; colonies whitish or cream, producing no fluorescent pigment; in Hugh & Leifson (1953) test no acid or gas is produced by fermentation, and sucrose, fructose and glycerol show no oxidative acid production (except for a few very slight changes); arginine test negative; nearly all strains failed to hydrolyse starch, casein or Loffler serum and very few gave H_2S from Kligler medium.

Phenon	2	3	4				
			4i	4ii	4iii	*M. lwoffi*	4, ungrouped
Number of strains	28	23	33	32	7	3	37
2 Characters not common to all strains							
Oxidative acid production from 5 sugars*	+	+	−	−	−	−	−
D. Oxidase	−	+(96)¶	+	−(97)	−	−	+(50)
D. Nitrate reduction	−(89)	+(91)	+(97)	−	−(71)	−	+(41)
Citrate	+(93)	−(78)	−	−(94)	−(57)	−	+(35)
D. Gluconate	+(68)	−(74)	−(91)	+(85)	+(86)	−	−(54)
Growth at 0°	−(75)	+	+(94)	+(97)	+(57)	−	+(77)
Growth at 37°	+(96)	−(87)	−(94)	−	−(57)	+	−(64)
D. Litmus milk (pH change)	ac (79)	uc (74), ac (26)	uc	uc	ac (71)	uc	uc (81), ac (14), alk (5)
D. Litmus milk (reduction)	−(64)	+(83)	+(85)	−(66)	+	+	+(49)
D. H_2S (cysteine)	−(64)	+(57)	−(82)	+(91)	+	−	+(73)
Gelatine liquefaction	−(71)	−	−	−(97)	−	−	−
Greening of blood plates	+(86)	−	−	−	−(71)	−	−(89)
D. Terramycin sensitivity (10 μg. tablets)	+(68)	+	+	+(50)	+	+	+(84)
D. Penicillin sensitivity (2·5 i.u. tablets)	−(93)	+	+(97)	+(87)	−	−(67)	+(73)
Penicillin sensitivity (i.u./ml.)	10− > 100	< 0·1	< 0·1	1–10 (few 1− > 100)	10− > 100 (few 1–10)	nt	< 0·1–20
DNA composition† (moles % GC)	37–41	44–45	44–46	40–43	37–40‡ 43–45§	nt	39–45

Abbreviations as in Table 10. D indicates differential characters for the subdivisions of Phenon 4.
* Glucose, galactose, arabinose, xylose and lactose.
† Mandel & Thornley (to be published).
‡ Four poultry isolates (MJT strains).
§ Three strains of *Mima*.
¶ Percentage of strains giving the indicated result.

in several properties. Strains 22 and 24, which formed one of these phenons, possessed polar flagella (Results, 2.2.1), did not produce alkalinity in litmus milk, gave 'very slow oxidation' of fructose, and produced a slow alkaline change in the arginine medium; all these properties distinguished them from the six strains of the other small phenon.

2.2.2. *Strains within the 72·5-phenon, but not included in Phenons 1–5*

These are listed in Table 8, and their properties may be mentioned briefly.

The two *Achromobacter* strains isolated from soil by Dr A. J. Holding, strains 7 (CB 11) and 8 (7A 14) were motile rods, flagella where determined (strain 8) being peritrichous. Strain 7 was closely related to Phenon 1, while strain 8 was equally similar to Phenons 1 and 5. Both strains differed from Phenon 1 in failure to grow on Paton medium and to produce H_2S in Kligler medium. While strain 7 gave similar sugar results to Phenon 1, strain 8 was less active, only glucose, galactose and xylose being oxidised, arabinose and glycerol giving the reaction described as 'very slow oxidation', and lactose, sucrose and fructose showing no change.

Strain 9 (A 16 of Moore & Pickett, 1960*a*) resembled both Phenons 4 and 5, but differed from both in its ability to liquefy gelatin and Loffler's serum, and the combination of other properties did not fit either phenon.

Strain 6 (EB/F64/100) was isolated from poultry, and was a Gram-variable motile rod with peritrichous flagella, forming cream-coloured colonies with spreading feathery outgrowths. This was originally thought to be a motile strain of *Achromobacter*, but from the computer results it showed little resemblance to any other motile *Achromobacter* strains, such as strains 7 and 8, and was not closely related to any of the phenons, although nearest to Phenons 4 and 5. It gave no acid from sugars, except for 'very slow oxidation' of glucose, was oxidase-negative, nitrate reduction-negative, did not grow on Simmons or Paton media at 0°, but grew at 37°, showed reduction but no pH change in litmus milk, and was penicillin-sensitive. Re-examination of the strain showed a large proportion of Gram-positive organisms, particularly when grown on solid medium. It was thought possible that this strain was close to some Gram-positive genus. It was examined by Dr T. Gibson (Edinburgh School of Agriculture), who found it to be a typical *Kurthia*.

Strain 19 (*Alcaligenes faecalis* NCTC 415) was not included in any phenon although closest to Phenon 5. When received, this strain showed a mixture of rough and smooth colonies, and was tested without single-colony isolation. Results included 'very slow oxidation' of four sugars and these were the main characters separating this strain from Phenon 5. When a single rough colony was separated, and re-tested (as 415A), acid was not formed from sugars and if these results had been included in the computation strain 415A would have been placed in Phenon 5; it was most like *A. faecalis* NCTC 8764, the representative strain of the phenon.

2.3. *Strains studied after completion of the computer survey*

Two strains reported to be similar to *Achromobacter liquefaciens* became available after the computer survey was made.

Achromobacter sp. NCIB 9650 is the strain described by Hendrie *et al.* (1964) as SKERMAN 381, which was studied by Skerman, Carey & MacRae (1958). Hendrie *et al.*'s observations, that this was a Gram-negative rod, motile by peritrichous flagella and

oxidase positive, were confirmed. In my tests, however, it did not liquefy gelatine, one of the few properties mentioned in Eisenberg's (1891) original description of *Bacillus liquefaciens*. It differed from the amended description of *Bergey's Manual* (1923) in producing alkalinity in litmus milk, instead of no change. Other properties were as follows: colony cream, rough, with a thin spreading edge, no acid from sugars, nitrate not reduced to nitrite, growth in Simmons medium, not on Paton medium, growth at 37°, no growth at 0°, penicillin-sensitive. When compared with strains used in the computer survey, it was most like the soil strain of *Alcaligenes*, CS8, from which it differed only in not reducing nitrate and being penicillin-sensitive. Strain CS8 had a high mean similarity to the rest of Phenon 5 (Table 6). *Achromobacter* sp. NCIB 9650 was also close to *Alcaligenes faecalis* NCTC 8764 and NCTC 415A, differing from both in three characters only; resemblances to strains in other phenons were very much lower, and it seems, therefore, that *Achromobacter* sp. NCIB 9650 should be classified as an *Alcaligenes* strain.

Tulecke *et al.* (1965) isolated an organism resembling *Achromobacter liquefaciens* as described by Eisenberg (1891) and by *Bergey's Manual* (1923); this strain is ATCC 15716. Tests used by the present author, where similar to those of Tulecke *et al.*, confirmed their results, with a few exceptions. Gram strains on organisms from broth cultures showed Gram-negative short rods, while those from nutrient agar cultures showed more than half the organisms to be Gram-positive. Also, litmus milk became alkaline, and in the conditions used here growth took place at 37°. Gelatine was liquefied and nitrate was not reduced, as reported by Tulecke *et al.*

The production of acid but no gas from glucose, sucrose and fructose was confirmed; this was shown by the Hugh & Leifson test to be a fermentative process. Fermentation of xylose, arabinose and galactose also took place, while glycerol gave acid in oxidative conditions only. This strain did not produce H_2S in Kligler or cysteine media, was oxidase-negative, gave traces of growth on Simmons medium, no growth on Paton medium, did not grow at 0° and was sensitive to penicillin and to terramycin. It was negative in effects on arginine medium, blood and starch plates and did not reduce litmus milk. Besides liquefying gelatine, it hydrolysed Loffler's serum and casein.

When compared with strains in the computer survey, ATCC 15716 differed from all strains by 14 characters or more. It was most similar to *Escherichia coli*, *Cellulomonas biazotea*, *Achromobacter* 7A 14, *Alcaligenes faecalis* NCTC 415 and *Achromobacter* EB/F64/100 (later shown to be *Kurthia*); from all these strains, ATCC 15716 differed by 14 or 15 characters, and for other strains in the survey, the differences were greater in number.

This organism ATCC 15716 is therefore very unlike any strains included in the survey. The large proportion of Gram-positive organisms in smears from solid media makes it seem possible that it has closer affinities to some Gram-positive genus than to Gram-negative or Gram-variable groups such as those studied here, and its sensitivity to penicillin is consistent with this.

2.4. *Penicillin sensitivity*

It was evident from the computer survey that sensitivity to 2·5 i.u. tablets of penicillin was a useful differential character (Tables 10, 11). Where inhibition zones were formed they varied considerably in size; for instance, both Phenons 4i and 4ii contained mostly strains sensitive to 2·5 i.u., but those in Phenon 4i all formed zones

larger than 3 mm, whereas in Phenon 4ii, only 32% of the sensitive strains produced zones > 3 mm.

The minimum inhibitory concentration (m.i.c.) of penicillin for selected strains was therefore estimated by the gradient plate method, and compared with the zone sizes formed by 2·5 i.u. tablets (Table 12). The m.i.c. was found to vary over a very wide range, from < 0·01 i.u./ml. to > 100 i.u./ml., that is, by a factor of more than 10^4. Almost the full extent of this range occurred among strains within Phenon 4.

It was noticeable that the range in m.i.c. was not continuous. Some strains, in

Table 12. *Penicillin sensitivity of selected strains: a comparison of results obtained with 2·5 i.u. tablets and by the gradient plate technique*

Phenon	Strain	Zone size* (mm.)	m.i.c.† (i.u./ml.)	Class‡
2	*Bact. anitratum* B 24	−F, −H	> 100	R
	Achr. lacticum NCIB 8208	−F, −H	50	R
3	MJT/F5/153	12F, 8H	0·07	VS
	MJT/F4/6/20	12Y, 11H	< 0·01	VS
	MJT/F4/6/17B	12Y, 8H	0·09	VS
4i	MJT/F5/239	15F, 8Y	0·02	VS
	MJT/F5/56	12F, 13H	0·06	VS
	MJT/F5/199A	11F, 15H	0·06	VS
	MJT/F4/11/5	4Y. 15H	0·07	VS
4ii	MJT/F5/122	2F, 4Y	5	I
	MJT/F5/111	2F, 5Y	4	I
	MJT/F5/139	1F	40	R
	MJT/F5/89	2F, 5H	4	I
	MJT/F5/284	3F, 3H	5	I
	MJT/F5/222A	8F	4	I
	MJT/F4/5/7	2Y	5	I
	MJT/F4/8/4B	4Y	5	I
	MJT/F4/5/2	−Y	4	I
	MJT/F4/21/5	−Y	8	I
	MJT/F4/17/7	6Y	4	I
	MJT/F4/2/6A	−Y	> 100	R
4iii	MJT/F5/14	−F, −H	70	R
	MJT/F5/5	−F, −H	30	R
	Mima 5936	−F, −H	5	I
	Mima Z 2	−F, −H	20	R
	MJT/F5/96	−F, −H	> 100	R
4 (ungrouped)	MJT/F5/195	11F, 10H	0·05	VS
	MJT/F5/130	1F	7	I
	MJT/F4/8/3	9Y, 14H	0·03	VS
	MJT/F4/21/9	−Y, −H	10	R
	MJT/F4/18/30	−Y, −H	20	R
	MJT/F4/3/16	−Y, −H	10	R
	M. lwoffi NCTC 5867	−F, 2H	14	R
	M. lwoffi 950/56	−F, 3H	3	I
	Alc. viscosus NCIB 8596	−F, 2H	3	I
5	*Alc. faecalis* NCTC 8764	−F, −H	> 100	R

* Radial distance from edge of pellet to edge of inhibition zone, on different media: F, FA; Y, YEA; H, HIA.

† m.i.c. = minimum inhibitory concentration, approximate value.

‡ m.i.c. < 0·1 i.u./ml., VS (very sensitive); 3–9 i.u./ml., I (intermediate); ≥ 10 i.u./ml., R (resistant).

Phenons 3, 4i, and the ungrouped part of Phenon 4 were very sensitive, with values for m.i.c. of < 0·1 i.u./ml. and zone sizes (with one exception) of 8 mm. or more, whichever basal medium was used. Among the other strains the values of m.i.c. ranged from 3 to > 100 i.u./ml., forming a continuous series. If these latter strains are classed as 'intermediate' or 'resistant' as indicated in Table 12, most intermediate strains gave small zones with 2·5 i.u. tablets, although some showed no zones, or variable results on different media. Nearly all resistant strains produced no zones. Most of the strains tested in Phenon 4ii were intermediate, with two exceptions which were resistant, while strains in Phenon 4iii were resistant except for one intermediate strain.

In practice, therefore, the test for sensitivity with 2·5 i.u. tablets (Shewan *et al.* 1954) reveals all the very sensitive strains and most of the intermediate strains; positive results in this test may be useful for screening unknown Gram-negative isolates, among which penicillin sensitivity is uncommon. For the separation of the phenons found here an additional routine test using a lower concentration of penicillin, which would detect only the very sensitive strains, would be most useful.

DISCUSSION

Section 1. General considerations

Representative strains of *Pseudomonas*, *Aeromonas*, *Escherichia*, *Cellulomonas* and *Arthrobacter* were excluded from the 72·5-phenon and were clearly shown to be not at all closely related to strains inside it. Non-pigmented glucose-oxidizing strains of *Pseudomonas* had been compared with non-motile coccoid rods from poultry (then called *Achromobacter*) in an earlier computer study by Thornley (1960*b*), and the two groups were quite distinct. It was therefore not thought necessary to include non-pigmented glucose-oxidizing pseudomonads in the present survey.

The classification of strains grouped within the 72·5-phenon remains to be considered. It must be remembered, however, that this phenon is not necessarily a taxonomic group, since strains within it may be more closely related to strains not studied in this survey than to other strains within the phenon. For instance, *Agrobacterium* strains grouped in Phenon 1 may well be closer to strains of *Rhizobium*, as would be suggested by the classification of *Bergey's Manual* (1957), than they are to strains in Phenons 2–5.

One of the most striking findings of this survey has been the separation of motile and non-motile strains into different phenons. Motile, Gram-negative rod-shaped bacteria were grouped into Phenons 1 and 5, or remained ungrouped, while non-motile Gram-negative or Gram-variable coccoid rods, cocci or short rods were grouped in Phenons 2, 3 and 4. Since the morphological features just mentioned formed only a small proportion of the information used by the computer, it follows that the phenons were also differentiated by other correlated characters such as those shown in Table 10.

The measurements of DNA composition by Mandel & Thornley (to be published) emphasized this distinction. The 46 strains studied in Phenons 2, 3 and 4 contained between 37 and 46 moles % guanine plus cytosine (GC) (Table 11). Twelve motile rod-shaped strains in Phenons 1 and 5, and motile *Achromobacter* strains (7, 8) had values ranging from 56 to 67 moles % GC.

In view of these facts, it seems desirable that strains in Phenons 2, 3 and 4 should be separated, in the scheme of classification to be adopted, from the motile strains in Phenons 1, 5 and ungrouped within the 72·5-phenon. Since representatives of *Alcaligenes* occur in Phenons 4 and 5, and *Achromobacter* strains occur in Phenons 1–4 and ungrouped within the 72·5-phenon, this entails division of the genera *Alcaligenes* and *Achromobacter*. Since both have motile type species, the generic names should be retained for the motile part of each genus (Discussion, 3.1; 3.3).

Section 2. Strains in Phenons 2, 3 and 4: the genus Acinetobacter

2.1. *The taxonomic position*

In view of the suggested generic division of the strains grouped in Phenons 2, 3 and 4 from those in Phenons 1 or 5, it would be logical, on the computer results only, to divide strains in Phenons 2, 3 and 4 into three distinct genera also. This is because the mean similarities between Phenons 2 and 3, 3 and 4, and 4 and 5 are very similar, when both computations are considered (Table 5), and this would suggest that each of these phenons should be given the same taxonomic rank. However, the author feels very doubtful about the validity of such a division, for several reasons.

From the computer results, when the characters separating phenons are examined carefully, it appears that Phenons 2 and 3 were separated from Phenon 4 mainly by the fact that strains in these two phenons oxidized five sugars (glucose, galactose, arabinose, xylose, lactose) while strains in Phenon 4 did not. This can be seen from Table 11. In the characters most useful for differentiating phenons, only sugar oxidation separated Phenon 3 and 4i. Penicillin sensitivity and DNA composition also showed marked similarity in these two phenons. Phenon 2 differed from 4ii and 4iii in sugar oxidation, and also an ability to grow in citrate medium and resistance to 2·5 i.u. of penicillin. In the latter two characters, it was closer to 4iii than to 4ii. It seems likely that the use of sugar oxidation as one feature in the original data would have resulted in strains now grouped in Phenons 2 and 3 being merged with strains in Phenon 4.

In the absence of biochemical evidence about the metabolism of sugars by the poultry isolates, it is impossible to guess whether a computer coding of one feature, five features, or some number in between would best represent the enzyme systems involved in acid production from these sugars. From the very high correlation of positive results for all five sugars, one would suspect that their metabolism is similar and therefore it would be undesirable to regard oxidation of the five sugars as five independent characters suitable for making the main basis of generic separations between Phenons 2, 3 and 4.

The values for DNA composition, mentioned earlier, support the idea that strains in Phenons 2, 3 and 4 may be more closely related to each other than they are to strains in Phenons 1 and 5.

It is suggested, therefore, that Phenons 2, 3 and 4 should be regarded provisionally as belonging to the same genus.

2.2. *Nomenclature and definition of the genus*

The correct generic name depends on the names of strains included in the three phenons, and their validity and order of publication. As already mentioned, both *Alcaligenes* and *Achromobacter* may be excluded from consideration.

Neisseria (Trevisan, 1885) is the oldest generic name. However, the one strain of *Neisseria* studied (*N. catarrhalis*, NCTC 3622) did not grow in some of the tests used, and therefore its inclusion in Phenon 4 is not well established. Besides this fact, the genus *Neisseria* consists entirely of coccoid cells, and therefore does not seem appropriate for a collection of strains ranging from cocci to short rods.

The name *Diplococcus mucosus* of von Lingelsheim (1906, 1908) is probably not appropriate for the strains in Phenon 2 (Discussion, 2.3); in any case, *Diplococcus* cannot be used as a generic name for this group because it is now regarded as a genus of the Tribe *Streptococceae* (*Bergey's Manual*, 1957).

Mima was mentioned as a genus of the Tribe Mimeae described by De Bord (1939); in 1942 *Herellea* was named by De Bord as another genus of Mimeae, and some further particulars were given. Henriksen (1963) discussed the inadequacy of the descriptions of both genera and various reasons why the names *Herellea* and *Mima polymorpha* var. *oxydans* should be considered illegitimate. He recommended that the specific epithet '*lwoffi*', from Audureau's (1940) description of *Moraxella lwoffi*, should be conserved against '*polymorpha*'; if it is decided to do this, the genus *Mima* will cease to exist. The present author considers that, in view of the lack of details in De Bord's descriptions, this would be the most satisfactory course.

Moraxella was proposed by Lwoff (1939) as a new genus for bacteria previously included in *Haemophilus* but not requiring X or V factors for growth. *Moraxella lacunata*, the Morax-Axenfeld bacillus, was the type species and needed unidentified growth factors present in serum, while *M. duplex*, with three varieties, would grow in peptone water. Later, additional species were proposed. Audureau (1940) described strains which would grow in an inorganic medium containing citrate and ethanol, and called these *M. lwoffi*. Four strains of *M. lwoffi* were included in Phenon 4 in the present survey. *Moraxella lwoffi* var. *glucidolytica* was described by Piéchaud & Second (1951) and later given specific rank as *M. glucidolytica* (Piéchaud *et al.* 1956). This species differed from *M. lwoffi* in producing acid from various sugars, and strains with properties similar to those described for *M. glucidolytica* were grouped in Phenon 2 in the present survey. If the classification of *M. lwoffi* and *M. glucidolytica* (or *anitrata*) in the same genus as *M. lacunata* and *M. duplex* be accepted, it would appear that *Moraxella* is the correct name for all strains in Phenons 2, 3 and 4.

However, this is the subject of controversy. The species mentioned above are similar in morphology (Murray & Truant, 1954), even when this is investigated in detail by electron microscopy (Ryter & Piéchaud, 1963). Besides the authors mentioned, Lwoff (1964) advocates inclusion of all these species in *Moraxella*, mainly for morphological reasons and also because of the observation of gliding motility in members of most of these species (Piéchaud, 1963).

Henriksen (1952) showed that the alleged *Moraxella* species could be divided into two groups on various properties; the species originally proposed by Lwoff being oxidase-positive and penicillin-sensitive, while *M. lwoffi* and *M. glucidolytica* (or *anitrata*) were oxidase-negative and penicillin-resistant. Henriksen concluded that the two latter should be excluded from *Moraxella*, and later (Henriksen, 1960) supported their classification as *Acinetobacter lwoffi* and *A. anitratum* by Brisou & Prévot (1954) and Brisou (1957). Steel & Cowan (1964) also favoured this solution, and pointed out that *A. lwoffi* and *A. anitratus* would be more correct forms of the names; their recommendations were followed by Pintér & Bende (1967). The present

study gives no direct evidence on this problem, because no strains of the oxidase-positive *Moraxella* species were included. It does, however, show that both oxidase-positive penicillin-sensitive strains and those with the converse properties, can be closely related, and therefore that these characters are not necessarily good ones for making generic divisions in this group of bacteria.

In the absence of direct evidence on the relationship between the bacteria studied here and *Moraxella lacunata*, it is suggested that the genus *Acinetobacter* of Brisou & Prévot (1954) should be used for the provisional classification of the bacteria of Phenons 2, 3 and 4. This would agree with the views of Henriksen (1960) and Steel & Cowan (1964) for '*anitratum*' and '*lwoffi*'. In addition to these species, which occur in Phenons 2 and 4, Brisou & Prévot placed *Achromobacter lacticum* (Phenon 2) and *Alcaligenes viscosus* (Phenon 4) in *Acinetobacter*. Their definition of the genus does not exclude any of the strains of Phenons 2, 3 and 4, and they mention 'short, coccoid forms are frequent', which fits the morphological properties of these phenons. In the amended definition of *Acinetobacter* by Steel & Cowan (1964), the character 'oxidase-negative' is added. This would have to be deleted to allow the inclusion of the strains of Phenons 3 and 4. Their addition of the property 'sugars oxidised or not attacked at all' is confirmed. The present study shows also that the arginine test was always negative in Phenons 2, 3 and 4 (Table 11).

The following revised definition is therefore suggested:

Acinetobacter Brisou & Prévot.

Gram-negative or Gram-variable, non-motile coccoid rods, short rods or cocci, often in pairs; able to grow aerobically on ordinary nutrient media without the addition of serum; colonies whitish or cream, producing no fluorescent pigment; catalase-positive; sugars oxidised or not attacked at all; arginine test negative.

The DNA composition found for 46 strains of *Acinetobacter* by Mandel & Thornley (to be published) ranged from 36 to 47 moles % GC.

Properties which varied among phenons included in *Acinetobacter* are listed in Table 11.

2.3. *The type species, Acinetobacter anitratus, as represented by the strains in Phenon 2*

The type species was designated *Acinetobacter anitratus* by Brisou (1957) and Steel & Cowan (1964). This species was originally described as *Bacterium anitratum* by Schaub & Hauber (1948) and Ferguson & Roberts (1950), and the latter authors also showed its identity with strains called B5W by Stuart, Formal & McGann (1949). Authentic strains used by all three authors (strains 1, 32–39 in Table 1) were included in the present survey, and were grouped in Phenon 2, together with one strain (strain 13, NCIB 9019) isolated by Brisou (1957) (Table 6).

As this phenon had a mean similarity of 84% and showed no signs of heterogeneity (Fig. 1), it is suggested that strains within the phenon should be regarded as representatives of the same species. This is supported by the fact that a comparable mean similarity value of 87% was found by Pintér & Bende (1967) for eight strains of *Acinetobacter anitratus*, most of which were originally identified by the criteria of Cowan & Steel (1965) as belonging to the same species.

2.3.1. *Nomenclature of the type species*

Besides strains originally called *Bacterium anitratum*, Phenon 2 included strains called *Achromobacter lacticum*, *Herellea*, *Diplococcus mucosus* and *Moraxella lwoffi*. Many previous authors have studied resemblances between strains with these names (except *Achromobacter lacticum*), and have indicated that some of them are synonyms. As the nomenclature of this group is confused, an account of the origins of the earlier names is given below. Comparative studies showing relationships between strains with these names (or between the definitions of the taxonomic groups) are listed in Table 13. As far as can be checked from descriptions, the work listed all relates to strains with the properties of Phenon 2.

Bacterium anitratum was well described in the early work mentioned above, and as original cultures have been preserved, the identity of new isolates with this species can be satisfactorily established. Unfortunately the generic name 'Bacterium' is invalid, but the specific name '*anitratum*' has been retained by many authors, and used in combination with *Achromobacter*, *Acinetobacter* or *Moraxella* (see Table 13). Henriksen (1963) considers that '*anitratum*' is the correct specific name, whatever generic name is finally adopted.

Moraxella lwoffi var. *glucidolytica* (or *M. glucidolytica*) was well described in the original studies of Piéchaud *et al.* (1951), which were amplified by Piéchaud *et al.* (1956). There is no doubt that the organisms they studied correspond in properties to organisms described as *Bacterium anitratum*; this has been shown by many comparative studies (Table 13).

Diplococcus mucosus was described by von Lingelsheim (1906, 1908) as a capsulated, Gram-negative coccus occurring in pairs or tetrads. None of his cultures is now available. Since that time, the name has been applied to strains with differing properties. The authors listed in Table 13 in reference to this organism, and the present author, studied strains previously identified as *Diplococcus mucosus*, but with properties similar to those of *Bacterium anitratum*. Seeliger (1952/53) considered that *D. mucosus* was the correct name for these organisms, and *Achromobacter mucosus* (von Lingelsheim, 1906) was used by Stenzel and Mannheim (1963) to designate a group of their own isolates with properties similar to *B. anitratum*. On the other hand, Cowan (1938) described isolates which resembled those of von Lingelsheim and which differed from *B. anitratum* in several respects, including the reduction of nitrates. Cowan considered that these organisms should be classified as *Neisseria mucosa*. Véron, Thibault & Second (1959) studied strains which resembled those of Cowan in reducing nitrate, but differed in other respects. They were cocci forming pairs and tetrads, were oxidase-positive and differed in sugar reactions and other properties from *B. anitratum*. Véron, Thibault & Second called them *Neisseria mucosa*, and later showed their serological relationship with strains of *Neisseria sicca*, but not with other species of *Neisseria* or with *B. anitratum* (Véron, Thibault & Second, 1961). It appears therefore that '*mucosus*' is not a suitable specific name for strains in Phenon 2.

Herellea was described by De Bord (1939, 1942) as a genus in the new tribe Mimeae together with the genera *Mima* and *Colloides*. No original strains are available. It was soon established that strains identified as *Herellea* by Deacon (1945) were similar in morphology and were serologically related to authentic strains of *Bacterium anitratum* (Ewing, 1949). Strains designated 'Herellea-like' by Aiken, Ward & King (1956)

Table 13. *Comparative studies of strains having the properties of Phenon 2*, with the nomenclature recommended by each author*

Reference	Original designation of strains studied: *Bact. anitratum* or B5W	*Moraxella lwoffi* var. *glucidolytica* or *M. glucidolytica*	*Diplococcus mucosus*	*Herellea vaginicola*	Strains isolated by the author listed	Name recommended in reference
Ewing (1949)	E, S†	—	—	E, S	E, S	In Tribe Mimeae of Parvobacteriaceae
Piéchaud *et al.* (1951)	E, S	E, S	—	—	E, S	*Moraxella lwoffi* var. *glucidolytica*
Brisou & Morichau-Beauchant (1952)	E	E	—	—	—	*Achromobacter anitratum*
Seeliger (1952–53)	E	—	E	—	E	*Diplococcus mucosus*
Brisou & Prévot (1954)	R†	R	—	—	—	*Acinetobacter anitratum* in Pseudomonadaceae
Aiken *et al.* (1956)	E, S	—	—	—	E, S	'Herellea-like' as descriptive term, temporary retention of Tribe Mimeae, to be placed in Pseudomonadaceae or Achromobacteraceae
Klinge (1959)	E	E	E	—	—	*Achromobacter anitratum, lwoffi, mucosus*
Henriksen (1960)	R	R	—	—	—	*Acinetobacter anitratum* in Pseudomonadaceae
Piéchaud (1961)	R	E	—	R	E	*Moraxella glucidolytica*
Courtieu *et al.* (1961)	E	—	E	E	E	*Moraxella* (provisional) *vaginicola*. Suggests separation of oxidase-negative species from *Moraxella* into another genus such as *Mima* or *Herellea*
Roscbury (1962)	R	R	—	R	—	*Mima vaginicola*, to be placed in same group as *Moraxella*
Stenzel & Mannheim (1963)	R	R	R	R	E	*Achromobacter mucosus*
Steel & Cowan (1964)	R	R	—	—	—	*Acinetobacter anitratus*
Lwoff (1964)	R	R	—	—	—	*Moraxella anitrata*

* As far as can be ascertained from descriptions.
† E, experimental studies; S, serology; R, review of data in literature.

were also biochemically and serologically similar to *B. anitratum*. Courtieu, Chassignol & Longeray (1961) and the present author included strains of *Herellea* obtained from Dr E. O. King in comparative surveys. Both studies confirmed that these strains possessed the properties of *B. anitratum*. However, Henriksen (1963) pointed out differences between the descriptions of *Herellea* given by De Bord (1942) and those of Deacon (1945) and later workers. Among several discrepancies, the fermentation of mannitol and dulcitol by De Bord's strains and no fermentation in Deacon's strains was one of the most important differences. Henriksen considered that the name *Herellea* was not applicable to strains, like those of Deacon, with the properties of *B. anitratum*, and that since strains corresponding to *Herellea* did not seem to be recognizable, this name should be rejected as a *nomen dubium*.

2.3.2. *Evidence from the present work*

The present study confirms earlier evidence (Table 13) that the same properties are shared by strains called *Bacterium* (or *Achromobacter* or *Acinetobacter*) *anitratum*, *Moraxella lwoffi* var. *glucidolytica* (or *Moraxella glucidolytica*), *Herellea* and *Diplococcus mucosus*. These species were all isolated from human sources, usually from pathological material but sometimes from healthy individuals (see Rosebury, 1962). A few cases of the isolation of similar organisms from saprophytic sites are quoted by Rosebury, including Billing's (1955) description of soap-tolerant strains (*Achromobacter anitratum* var. *saponiphilum*) from face-flannels and sponges.

The present investigation showed for the first time that strains from slimy milk, called *Achromobacter lacticum* and agreeing with the description of this organism in *Bergey's Manual* (1939, p. 519), were also indistinguishable from *Bacterium anitratum*. One of these strains, NCIB 8208, was the second highest in order of mean similarity to the other strains in Phenon 2, with a value of 88·0% (Table 6). The highest mean similarity value, 88·5%, was shown by *B. anitratum* B25 (B5W99), one of Ferguson & Roberts' (1950) strains. The positions of NCIB 8208 and NCIB 8209 in Table 6 indicate that both these strains had properties typical of Phenon 2.

Of the 110 poultry isolates studied, only one strain (MJT/F5/211) fell into Phenon 2, to which its mean similarity was low (Table 6); its properties were not typical of the phenon, as it reduced nitrate and was sensitive to penicillin. Gardner (1965) studied the microflora of fresh meat, and of 91 isolates called *Achromobacter* which were similar in general properties to the poultry isolates, only one strain had properties similar to those of Phenon 2.

2.4. *Phenons included in Acinetobacter but not regarded as species*

The close resemblance between strains in Phenon 3 and those of Phenon 4i (Discussion, 2.1) suggests that it would be premature to assign specific status to Phenons 3 and 4, since the study of additional characters might in future cause the rearrangement of these phenons.

2.4.1. *Phenon 3*

This contains only poultry isolates and one strain from fish. No strains of human origin with the same properties (Table 11) have been described. Perhaps the closest resemblance is to *Moraxella saccharolytica*, described by Flamm (1956) on the basis of one strain, and discussed by Henriksen (1960). This strain had the morphological

properties typical of a *Moraxella*, was oxidase-positive, penicillin-sensitive and decomposed some sugars to acid products. However, it was resistant to tetracyclines, while the strains in Phenon 3 were sensitive to terramycin (oxytetracycline).

Among bacteria of non-medical significance, strains with similar morphological properties were mentioned by Shewan *et al.* (1960). These were penicillin-sensitive and either oxidized or did not attack glucose in the Hugh & Leifson test. The oxidase test was not applied to these strains, but a similar group was described as oxidase-positive by Hendrie *et al.* (1964); as one of Dr Shewan's strains was included in Phenon 3, there is little doubt that his glucose-oxidizing strains are similar to the poultry isolates of Phenon 3. Shewan *et al.* (1960) included this group in *Achromobacter*, and Hendrie *et al.* (1964) retained this term, but suggested that the genus *Acinetobacter* of Brisou & Prévot (1954) might be appropriate.

Among the 91 strains from fresh meat identified as *Achromobacter*, Gardner (1965) found four strains which were oxidase-positive, sensitive to 2·5 i.u. penicillin and oxidized glucose in the Hugh & Leifson test. As far as they have been described, these were similar to the poultry isolates of Phenon 3.

2.4.2. *Phenon 4*

This contained 93 poultry isolates and 17 named strains including *Mima*, *Moraxella*, *Alcaligenes viscosus* and (possibly) *Neisseria catarrhalis*, and the properties found within the phenon and its subdivisions are listed in Table 11.

Phenon 4i contained an oxidase-positive strain of *Mima*, strain Z4. It has been stated (Henriksen, 1952; Murray & Truant, 1954) that *Mima polymorpha* var. *oxidans* is indistinguishable from *Moraxella duplex* var. *nonliquefaciens*, and Catlin & Cunningham (1964*b*) showed transformation between strains with these two names. It appears therefore that Phenon 4i may be similar to the latter organism. Besides the oxidase-positive nature of the strains, their very high penicillin-sensitivity fits in with the results of Henriksen (1952) for this species.

Phenon 4ii contained no named strains.

The significance of Phenon 4iii is not clear, as it was a very small group (seven strains), did not appear in the second computation, and contained two sets of strains with significantly different GC content of the DNA, which was 37–40 moles % for the four poultry strains and 44–45% for three *Mima* strains (Mandel & Thornley, to be published).

The portion of Phenon 4 which remained ungrouped into smaller phenons consisted of 27% of the poultry isolates and 59% of the named strains. Their failure to fall into the small groups was due to the possession of different combinations of the properties shown in Table 11, but in general the correlation between results in the oxidase test and reduction of nitrate shown in Phenons 2, 3, 4i, 4ii and 4iii held good for most of the ungrouped strains also. Only 11% of the poultry isolates and 4 of the 17 named strains in Phenon 4 did not show this correlation.

Although the oxidase test is useful for differentiation of genera among other groups of bacteria, this does not seem to apply here, because Phenons 4i and 4ii, which differed in this character, were linked at 90% similarity. This seems much too close for division into different genera.

The synonymity between certain species of *Moraxella* and *Mima* was first noted in connection with the oxidase-positive strains already mentioned. From the investi-

gations listed in Table 13, which showed the similarity of strains previously identified as *Moraxella glucidolytica* to those identified as *Herellea*, it followed that strains called *Moraxella lwoffi* should be identical with oxidase-negative strains of *Mima polymorpha*, since both had already been shown to resemble the sugar-oxidizing strains in all but sugar reactions (Piéchaud *et al.* 1951; Aiken *et al.* 1956).

The present study shows that isolates received as *Moraxella lwoffi* were closely related to those received as *Mima*, since all were included in Phenon 4. Apart from the oxidase-positive *Mima* Z4, six oxidase-negative strains called *Mima* and four called *Moraxella lwoffi* were studied, and considerable variation in properties was found among these ten strains. Pintér & Bende (1967) studied a group of 31 strains of *Acinetobacter lwoffi*, including 28 of their own isolates and NCTC 5866 (strain 63 of the present work); the 31 strains formed a closely related group with a mean similarity of 82%. The properties of these strains were not quoted, but in the present work, NCTC 5866 formed part of the group of three strains of *Moraxella lwoffi*, for which properties are given in Table 11. A further comparison of more human isolates with poultry strains would be useful to show whether this very small group represents a large proportion of strains of *M. lwoffi*, and whether these would form a group distinct from the groups of poultry isolates.

Neisseria catarrhalis NCTC 3622 was also placed in Phenon 4, but this was not very well established, because of its failure to grow in some tests. Nevertheless, a relationship with strains similar to *Moraxella* would not be surprising in view of Catlin's (1964) observation of transformation between *N. catarrhalis* and *M. nonliquefaciens*. The DNA of seven strains of *N. catarrhalis* contained between 41 and 44·5 moles % GC (Catlin & Cunningham, 1962*a*), a value within the range shown by Phenon 4 (Mandel & Thornley, to be published; Table 11). The observation of division into tetrads by one of the poultry isolates (Pl. 2, fig. 6) is a property previously thought to be characteristic of *Neisseria* (Murray & Truant, 1954; Piéchaud, 1961).

The similarity of *Alcaligenes viscosus* to *Moraxella lwoffi* was noted by Brisou & Prévot (1954), who placed them both in the genus *Acinetobacter*, and by Billing (1955), and is confirmed here by the inclusion of two *A. viscosus* strains in Phenon 4.

Bacteria which were morphologically similar to strains in Phenon 4 have been described as occurring on poultry, meat and fish, particularly during aerobic storage at low temperatures; several references are quoted in the Introduction, and all the authors called these isolates either *Achromobacter*, *Alcaligenes* or *Achromobacter-Alcaligenes* group. Buttiaux (1961) described *Achromobacter* strains as non-motile coccoid rods which did not produce acid from sugars and might be oxidase-positive or oxidase-negative; these are probably similar to the poultry isolates of Phenon 4. Buttiaux suggested a future generic separation of oxidase-positive from oxidase-negative strains, but the present results do not support such a separation.

Sulzbacher (1950) described isolates from frozen pork, some of which were non-motile very short oval rods, probably belonging to *Achromobacter*; one of his isolates (*Achromobacter* 25A2, strain 12) was included in Phenon 4 of the present survey. The strains of non-motile *Achromobacter* (or *Acinetobacter*) of Hendrie *et al.* (1964) were described as mostly oxidase-positive, and one of their strains (*Achromobacter* 131, strain 10) was placed in Phenon 4. Although both strains, 25A2 and 131, were found to be oxidase-positive, neither was included in Phenon 4i as the combination of other properties was not typical of this small phenon (Table 11). Of Gardner's (1965) 91

isolates of *Achromobacter* from fresh beef, 83 strains were inert with glucose, so would appear to be similar to Phenon 4. These 83 strains included both oxidase-positive and oxidase-negative, penicillin-sensitive and penicillin-resistant strains; 41 strains were oxidase-positive and penicillin-sensitive.

It is clear that isolates from a wide variety of protein foods are similar to the bacteria included in Phenon 4.

Section 3. Other genera represented in the survey

3.1. *Alcaligenes*

Of the strains received as *Alcaligenes*, two non-motile oxidase-negative strains of *Alcaligenes viscosus* (NCIB 8154, NCIB 8596) were included in Phenon 4 and have been dealt with as *Acinetobacter*. The other eight strains were all oxidase-positive and motile under suitable conditions, and included two strains with polar flagella and five with peritrichous flagella (Results, 2.2.1.1).

Alcaligenes faecalis, the type species, is described as peritrichously flagellate, and two named strains of this species (21, NCTC 655; 20, NCTC 8764) had this property and were grouped with four other strains (of which three had peritrichous flagella) in an 87·5-phenon. This included two strains from soil (29, 30) which were closely related to the rest of the phenon, and strain 25, NCTC 8582, the type species of *A. denitrificans*. This species was separated from *A. faecalis* by Leifson & Hugh (1954) on the basis of gas production from nitrate, but the close relationship shown here (Fig. 5) provides no evidence to support species separation. Strain 26, NCTC 3233, may have been originally mistakenly identified as *A. viscosus*; the two other strains of this name (NCIB 8154, NCIB 8596) which were included in Phenon 4 corresponded more closely to the descriptions of the species by Long & Hammer (1936) and Abd-el-Malek & Gibson (1952). The strain *Achromobacter* sp. NCIB 9650, studied after the computing was done, was also very similar to several strains in the 87·5-phenon, and should be classified as an *Alcaligenes*.

The properties of the six strains in the 87·5-phenon agreed with those described for *Alcaligenes faecalis* in *Bergey's Manual* (1957), and in addition, they were oxidase-positive and citrate-positive, as quoted by Thibault (1961) for peritrichously flagellate strains. These properties are included in the results for the whole of Phenon 5 listed in Table 10. However, a more extensive study of strains of *A. faecalis* is reported by Board (1965).

Both polarly flagellate strains (22 *Alcaligenes faecalis* NCTC 8769; 24 *A. bookeri* NCIB 8155) showed several resemblances, including the late positive result in arginine, to Hugh & Ikari's (1964) neotype strain of *Pseudomonas alcaligenes* (ATCC 14909, NCTC 10367). However, the DNA composition of strains 22 and 24 was 62–63 moles % GC (Mandel & Thornley, to be published) while that of ATCC 14909 was 66 moles % GC (Mandel, 1966). This suggests that strains 22 and 24 may be more similar to *P. pseudoalcaligenes* (Stanier, Palleroni & Doudoroff, 1966), which contained 63 moles % CG (Mandel, 1966).

The finding of both polarly and peritrichously flagellate strains among bacteria previously identified as *Alcaligenes* has been reported many times; for instance, by Nyberg (1935), Türck (1952), Moore & Pickett (1960*b*) and Thibault (1961). The present work indicates a few differences in biochemical properties between the two groups (Results, 2.2.1.2), but many more could probably be found by the application

of tests characteristic of *Pseudomonas alcaligenes* and *pseudoalcaligenes* (Stanier *et al.* 1966) and of *Alcaligenes faecalis* (Board, 1965).

Several authors have recommended that the genus *Alcaligenes* should be abandoned, and that strains formerly called *Alcaligenes* should be placed in *Achromobacter*. Brisou & Prévot (1954) separated non-motile strains as *Acinetobacter*, and motile strains, including both polarly and peritrichously flagellate types, were regarded as *Achromobacter*. Hendrie *et al.* (1964) agreed with the abolition of *Alcaligenes*, and the separation of non-motile strains, but would include only peritrichous strains in *Achromobacter*. Moore & Pickett (1960*a*, *b*) wished to abolish *Alcaligenes* and include both non-motile and peritrichous strains of *Achromobacter*. The results of the present work showed the existence of a well-defined group of six strains with the properties of *Alcaligenes faecalis*, including peritrichous flagellation. Since this is the type species of *Alcaligenes*, the position of this genus seems much more satisfactory than that of *Achromobacter*, in which the existence of the type species is still doubtful. The retention of the genus *Alcaligenes* for strains resembling *A. faecalis* therefore seems desirable.

3.2. *Agrobacterium*

Only three named strains of *Agrobacterium* were included in the survey, and these were grouped in Phenon 1 together with strain 2, *Achromobacter hartlebii* NCIB 8129. This suggests that strain 2 should be considered for possible inclusion in *Agrobacterium*.

In the present survey, the oxidative production of acid from sucrose and fructose, and the formation of H_2S in Kliger medium, differentiated Phenon 1 from all strains in Phenons 2–5, while the positive oxidase test for Phenon 1 was useful for differentiation from the oxidase-negative groups. However, it is not known whether these properties are widespread among *Agrobacterium* strains. The 3-ketoglycoside test of Bernaerts & De Ley (1963), which is claimed to give positive results only for strains of *Agrobacterium*, was unfortunately not included in the survey, as the tests were completed before its publication.

3.3. *Achromobacter*

Most of the strains received as *Achromobacter* proved to be non-motile coccoid rods, which were grouped in Phenons 2, 3 and 4, and have been placed provisionally in the genus *Acinetobacter*.

Of the strains found to be motile, *Achromobacter hartlebii* NCIB 8129 was included in Phenon 1, and may be suitable for classification as *Agrobacterium* (Discussion, 3.2).

The two soil strains isolated by Dr A. J. Holding differed, *Achromobacter* CB 11 (strain 7) being much like Phenon 1 and *Achromobacter* 7A14 (strain 8) being equally similar to Phenons 1 and 5 (Table 8, Results 2.2.2.). These strains could be considered as motile Achromobacters, but the resemblance to Phenon 1 emphasizes the need to establish whether any major differences exist between *Agrobacterium* strains and motile glucose-oxidizing strains classified as *Achromobacter*. Of the differential characters used by Holding (1960), only a growth requirement for organic nitrogen separated the two groups, strains with this requirement being placed in *Achromobacter*.

The strain isolated by Tulecke *et al.* (1965), *Achromobacter liquefaciens* ATCC 15716, may well be similar to that originally described by Eisenberg (1891), but its relation to other Gram-negative strains suitable for inclusion in *Achromobacter* remains to be

proved; it has many differences from all strains in this survey, and it may be suggested (Results, 2.3) that it is more closely related to some Gram-positive genus.

The existence of a group of motile strains suitable for classification as *Achromobacter* has therefore not been established, although Holding's two soil strains may be in this category. However, there are further motile strains available in culture collections which were not studied here. Since the types species, *A. liquefaciens*, is described as motile, it seems best to reserve the genus *Achromobacter* for any motile strains which may prove to be suitable for inclusion.

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EXPLANATION OF PLATES

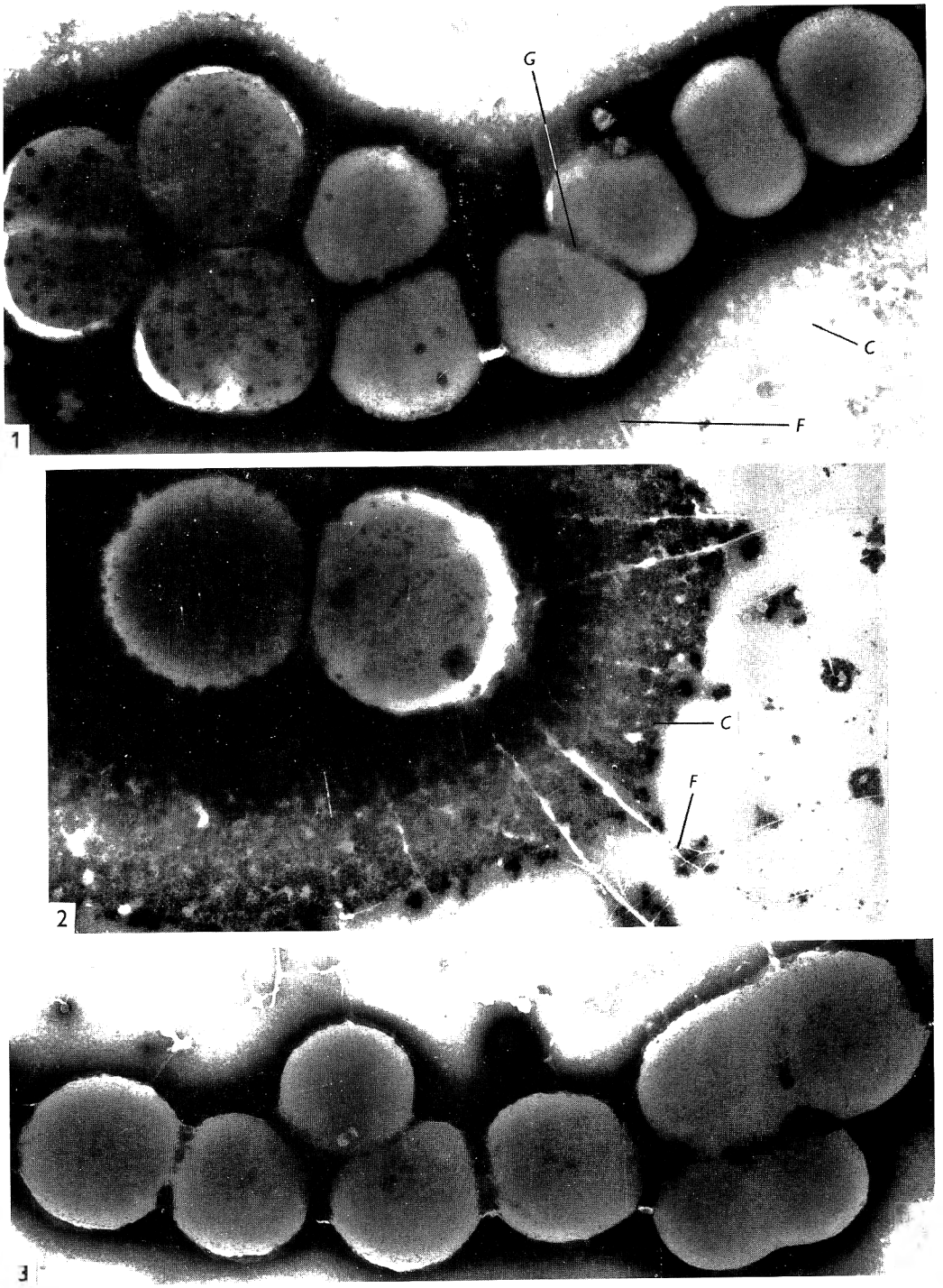
PLATE 1

Fig. 1–3. Electron micrographs of intact cells, negatively stained with potassium phosphotungstate.

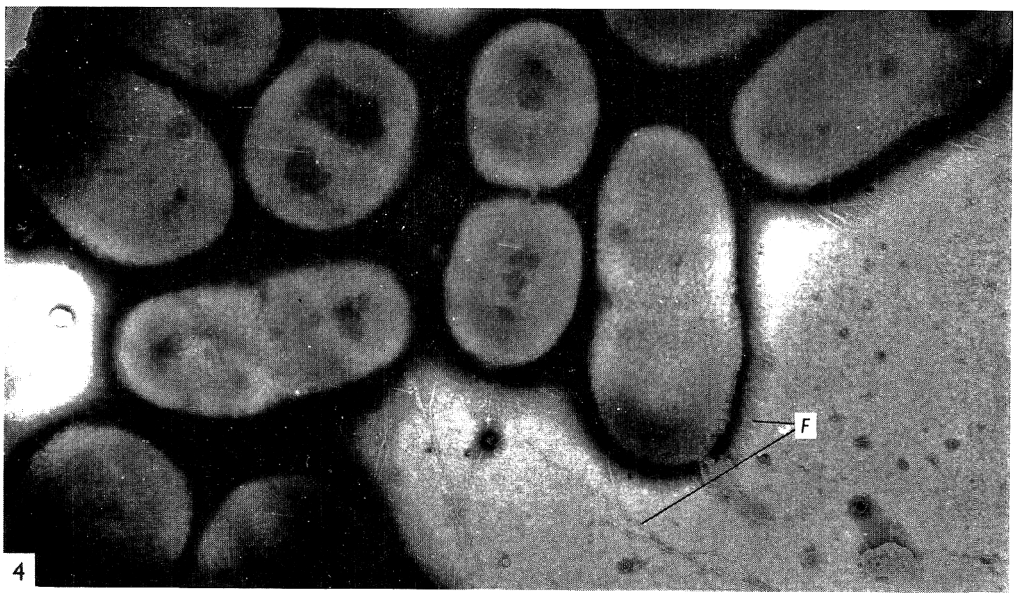
Fig. 1. Strain MJT/F5/158, the representative strain of Phenon 3. Capsular material (*C*) and fimbriae (*F*) are present, and grooves (*G*) show the positions at which cell division is proceeding. × 24,000.

Fig. 2. Strain MJT/F4/8/3 in Phenon 4, which consists of cocci in pairs with a very thick capsule (*C*) and long fimbriae (*F*). × 28,000.

Fig. 3. *Alcaligenes viscosus* NCIB 8596, in Phenon 4. The cocci are arranged in chains. × 24,000.



(*Facing p.* 256)



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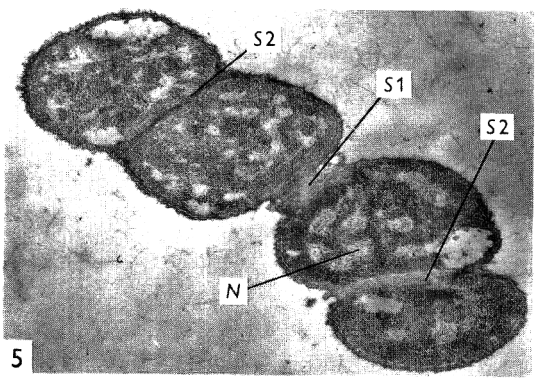

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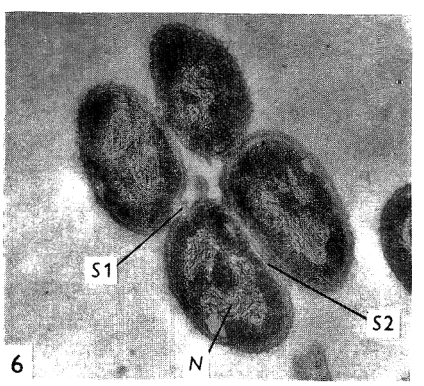

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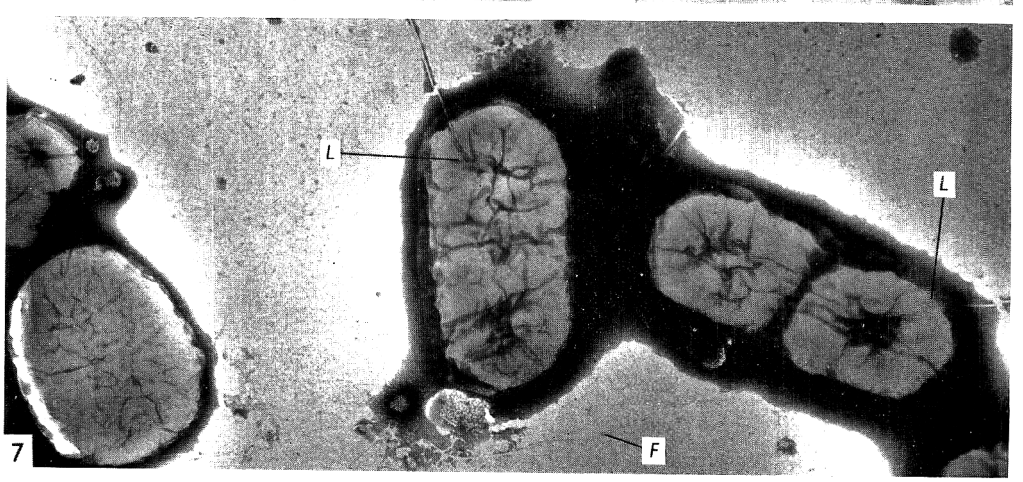

7

PLATE 2

Fig. 4 and 7. Electron micrographs of intact cells, negatively stained with potassium phosphotungstate.

Fig. 5. and 6. Electron micrographs of thin sections of strain MJT/F5/199A in Phenon 4, after different periods of growth in aerated broth.

Fig. 4. Strain MJT/F5/5, in Phenon 4iii. The cells are coccoid rods and short rods, with fimbriae (*F*). × 28,000.

Fig. 5. Cells from a 10-hr. culture, forming a short chain. The septum marked *S*1 was formed earlier, and later divisions (*S*2) are taking place in a plane parallel to the first. N-nuclear material. × 20,000.

Fig. 6. Cells from a 24 hr. culture, forming a tetrad. The remaining part of an earlier septum (*S*1) shows a division plane perpendicular to that of the later-formed septum, *S*2. N-nuclear material. 20,000.

Fig. 7. Intact cells of *Alcaligenes faecalis* NCTC 655, in Phenon 5, consisting of short rods and coccoid rods. Two flagella with lateral attachment (*L*) are shown, and fimbriae (*F*) are present. The surface appearance of this strain, in which the phosphotungstate has penetrated well-marked grooves or wrinkles, differs from that of the non-motile cocci or coccoid rods in Figs. 1–4. × 28,000.

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The Autolysis of *Aspergillus terreus* in a Physiologically Acid Medium

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SUMMARY

The behaviour of certain intracellular components contained in the mycelium of *Aspergillus terreus* during autolysis were studied. *Aspergillus terreus* grown in an acid medium underwent an 'acid autolysis' in which a decrease in mycelial dry weight amounting to 14·6% occurred. The content of fat present in the autolysing mycelium continuously decreased throughout the pre-autolytic and autolytic stages. The concentration of free glucose in the mycelium continuously increased at the beginning of autolysis, whereas xylose soon disappeared when autolysis began. At the end of the log phase and during the pre-autolytic stages the content of mannitol sharply decreased, at the beginning of autolysis its concentration decreased at a slower rate, being constant at the end of the autolytic period studied. Fourteen different amino acids were found in the autolysing mycelium of *A. terreus*. Eighty-six % of the maximum concentration of these free amino acids disappeared from mycelium before autolysis; 81% of the remainder during autolysis.

INTRODUCTION

Studies on the chemistry of the autolytic phase of growth in filamentous fungi grown in physiologically alkaline and acid media have been reported only on three occasions for four different moulds: *Aspergillus niger* (Behr, 1930); *Cunninghamella elegans* (Schmidt, 1936); *Mucor adventitius* and *Absidia glauca* (Ritter, 1955). In these studies the behaviour of certain nitrogenous compounds during autolysis, in two types of medium (NO_3^- or NH_4^+ as N source) were described. However, very few papers have presented quantitative data on the variation of the amount of intracellular free sugars and free amino acids contained in autolysing mycelium in cultures on an acid medium.

Continuing our work on the chemistry of autolysis in fungi we reported in a previous paper (Lahoz, 1967) the behaviour of *Aspergillus terreus* grown in an alkaline medium, during autolysis. The present work describes the qualitative and quantitative chemical changes in lipids, free sugars, mannitol and free amino acids occurring in *A. terreus* grown in an acid medium during the autolytic phase of growth.

METHODS

Organism. Aspergillus terreus Thom (our collection no. 2426) was used as a physiological model in the present studies with a liquid medium.

Chemicals. Chemicals used in the preparation of the culture medium were of

analytical purity, all obtained from Probus, S. A. (Barcelona, Spain). Other chemicals used were either from the British Drug Houses Ltd. (Poole, Dorset, England) or from Sigma Chemical Co. (St Louis, Mo., U.S.A.).

Culture medium and cultivation of the organism. The organism was grown in static cultures in a modified Czapek-Dox medium, in which the source of nitrogen, $NaNO_3$, was replaced by its equivalent amount of $(NH_4)_2SO_4$, thus leading to an 'acid autolysis'. The medium had the following composition: glucose (anhydrous) 50 g.; $(NH_4)_2SO_4$ 1·55 g.; KCl 0·5 g.; $MgSO_4.7H_2O$ 0·5 g.; KH_2PO_4 1·0 g.; $FeSO_4.7H_2O$ 0·01 g.; distilled water 1000 ml. Fifteen l. of this medium were prepared and distributed in 100 ml. portions in Jena glass conical flasks of 300 ml. capacity. The flasks were plugged with non-absorbant cotton wool and sterilized by steaming on three consecutive days, for 30 min. each day. When at room temperature, a heavy spore suspension of *Aspergillus terreus* was inoculated (about 2 ml.) into each flask. These flasks were then incubated at 24–25° in the dark.

Samples (5 flasks) were withdrawn at intervals from the incubator. The mycelia were separated from the culture fluids by filtration. These culture fluids from 5 flasks were pooled and diluted with distilled water to the original volume. The mycelia were washed free from culture fluid with distilled water, cut into small pieces and dried to constant weight at 60–70°.

Fractionation of mycelium. Weighed samples of powdered mycelium harvested at different times were exhaustively extracted with light petroleum (b.p. 50–70°). The solvent was removed from the extract by distillation and the crude lipids left in the flask dried in vacuum to constant weight; this constituted fraction 1. The defatted mycelium was carefully removed from the percolator, dried at 80°, weighed and extracted twice by cold (22°) distilled water (100 ml./g. dry defatted mycelium) with mechanical stirring for 1 hr. The cold-water extracts were combined and stored at −10° until needed (fraction 2). To test for completeness of extraction the residue was a third time extracted and the resulting extract concentrated under reduced pressure and a sample of it chromatographed to examine for sugars and amino acids.

One hundred ml. of fraction 2 were concentrated in vacuum at 70° on a water bath to a final volume of 10 ml. Portions (0·5 or 0·7 ml.) of this concentrate were used for chromatographic analysis of sugars and mannitol. The remainder of each concentrate was kept at −10°.

The determination of total nitrogen, fat, total free reducing substances, free sugars and mannitol were as described previously (Lahoz, Reyes & Beltrá, 1966).

Estimation of individual amino acids. One hundred ml. of fraction 2 were concentrated to dryness at 60–70° under reduced pressure and extracted with 50 ml. of the ethanol + water + conc. HCl (95 + 4·5 + 0·5, by vol.) mixture described by Baliga, Krishnamurthy, Rajagopalan & Giri (1955). This extract was concentrated and the amino acids separated and identified by paper chromatography using as solvent the butan-1-ol + acetic acid + 96% (v/v) ethanol + water (40 + 10 + 10 + 20, by vol.) mixture on Whatman no. 1 filter paper. The papers were developed by descending chromatography in three successive periods of 24 hr runs, each time air-dried and newly introduced into the tank for a total time of 72 hr. Elution of the spots and determination of the amount of each of the individual amino acids was done by the method of Giri, Radhakrishnan & Vaidyanathan (1953) as modified by Kay, Harris & Entenman (1956). Readings were done in a Beckman spectrophotometer, model B at 575 mμ. Standards were

prepared from leucine of analytical purity obtained from Sigma Chemical Co. (St Louis, Mo., U.S.A.). Results are expressed as g. amino acid/100 g. dry mycelium.

Total phosphorus. This was determined by a modification of the method of Fiske & SubbaRow (1925).

RESULTS

Autolysis of *Aspergillus terreus* in a medium containing NH_4^+ as N source took place at about pH 2 (Table 1). Dry weight of mycelium (Table 2) reached a maximum, 1·0824 g./flask, at the 18th day of incubation and was 0·9242 g./flask at the 150th day

Table 1. *The autolysis of Aspergillus terreus in cultures in an acid (pH 2) medium at 24–25° in the dark. pH values residual glucose, nitrogen and phosphorus of the culture fluid at various times of incubation*

Time of incubation (days)	Time of autolysis (days)	pH value	Residual glucose (g./100 ml.)	Nitrogen (mg./100 ml.)	Phosphorus (mg./100 ml.)
6	.	1·8	2·950	0·00	15·5
7	.	1·9	2·700	0·00	15·5
8	.	1·8	1·440	0·00	14·7
9	.	1·8	1·360	0·00	14·3
10	.	1·9	0·790	0·00	13·3
11	.	2·0	0·590	0·00	13·5
12	.	1·9	0·310	0·00	14·3
13	.	1·9	0·300	0·00	15·8
14	.	2·0	0·200	0·00	17·5
15	.	2·1	0·140	0·00	18·8
18	0	2·1	0·053	0·00	20·5
24	6	2·0	0·060	5·10	21·3
30	12	2·1	0·054	8·10	.
42	24	2·1	.	8·76	.
54	36	.	.	8·63	.
66	48	2·0	.	8·00	.
78	60	.	.	6·91	.
90	72	.	.	10·70	.
102	84	.	.	9·00	.
114	96	.	.	8·90	.
126	108	.	.	9·50	.
138	120	1·9	.	9·20	.
150	132	.	.	8·89	.
162	150	.	.	6·80	.

of autolysis. The loss in mycelial dry weight amounted to 14·6% during this autolytic period. The criterion of autolysis we have adopted throughout this work was the loss in mycelial weight. According to that, autolysis began on the 19th day of incubation. Carbon source (glucose) initially present at 5 g./100 ml. medium, reached an average constant value 0·055 g./100 ml. culture filtrate (Table 1), after the zero day of autolysis. A progressive increase in the content of total P in the culture filtrate during the pre-autolytic stages occurred (Table 1). Nitrogen was not detected in the culture filtrate between the 6th and the 18th days of incubation; at the 24th day 5·1 mg. N/100 ml. were present.

The percentage of nitrogen in autolysing mycelium of *Aspergillus terreus* (Table 2) grown with $(NH_4)_2SO_4$ as N source decreased slowly through the whole period of incubation, reaching a minimum constant value, 2·1%, at the 96th day of autolysis. The amount of total P present in mycelium during the incubation period here studied

decreased by about a half (54%) with respect to its initial maximum content, whereas the disappearance of P during the first 24 days of autolysis amounted to 75% (Table 2). The highest amount of fat present in mycelium of *A. terreus* was observed at the 9th day of incubation. From this stage on the content of fat continuously decreased during the whole cycle of autolysis, being 4·9 g./100 g. dry mycelium (Table 2), the final value observed at the end of the autolytic period (150 days).

Table 2. *The autolysis of Aspergillus terreus in cultures in an acid medium at 24–25° in the dark*

Amounts of nitrogen, phosphorus and fat in the mycelium at different times of incubation.

Time of incubation (days)	Time of autolysis (days)	Mycelium dry weight (g./flask)	N (%)	P (%)	Fat (g./100 g. dry mycelium)
6	.	0·6695	4·7	.	.
7	.	0·7988	3·4	0·96	19·0
8	.	0·8907	.	0·96	.
9	.	0·9264	2·8	0·92	21·6
10	.	0·9540	.	0·94	.
11	.	1·0090	.	0·87	20·5
12	.	1·0380	.	0·74	.
13	.	1·0652	.	0·68	.
14	.	1·0589	2·6	0·53	.
15	.	1·0770	.	.	15·8
18	0	1·0824	.	0·44	.
24	6	1·0764	.	0·29	13·6
30	12	1·0715	2·6	0·19	.
42	24	1·0661	.	0·11	13·5
54	36	1·0519	.	0·11	.
66	48	0·9796	.	.	10·0
78	60	0·9769	2·3	.	.
90	72	0·9625	.	.	9·4
102	84	0·9554	.	.	.
114	96	0·9677	2·1	.	8·8
126	108	0·9784	.	.	.
138	120	0·9628	2·1	.	.
150	132	0·9548	.	.	.
162	150	0·9242	2·1	.	4·9

Behaviour of the total free reducing substances, free sugars and mannitol. At the beginning of autolysis the amount of water-extractable reducing substances contained in the mycelium of *Aspergillus terreus* stayed nearly at a minimum (Fig. 1). At 36 days incubation the content of these materials were the highest observed, reaching to 1175 mg./100 g. dry mycelium. After that time the amount of total free reducing substances sharply decreased to values fairly constant in the range 300–400 mg./100 g. dry mycelium. The behaviour of free glucose showed a very similar pattern (Fig. 1) to that of total free reducing substances. Glucose accounted for an average of 70% of the total reducing power observed for the aqueous extracts. From the 40th day of incubation xylose (Fig. 1) tended to decrease. No appreciable amount of xylose was detected between 66 and 78 days of incubation.

During the phase of growth (9 days) mannitol was present in mycelium of *Aspergillus terreus* at (Fig. 2) 6·1 g./100 g. dry mycelium. As incubation proceeded this amount decreased sharply to the 18th day of incubation (zero days of autolysis) when 2·5 g.

mannitol/100 g. dry mycelium were present. At the beginning of autolysis its concentration decreased at a slower rate; between the 72 and 84 days the concentration of mannitol remained constant.

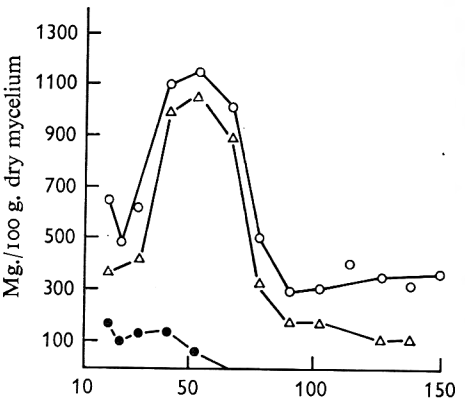

Fig. 1

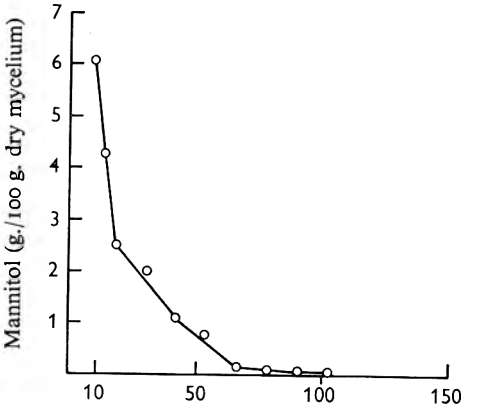

Fig. 2

Fig. 1. Changes in the concentration of total free reducing substances ○; free glucose △; xylose ●, in autolysing mycelium of *Aspergillus terreus* grown in a medium with NH_4^+ as N source.

Fig. 2. Content of mannitol in autolysing mycelium of *Aspergillus terreus* grown in an acid medium.

Behaviour of the amino acids and its variation with age

The nature and amounts of free amino acids released from mycelium harvested at different times is shown in Table 3. Thirteen different amino acids were identified; one, present in relatively small amount, was not identified (unknown). The total content of these free amino acids increased between 7 and 9 days of incubation to a maximum. Thereafter the general picture was of decreasing concentration both at the end of the log phase and during the pre-autolytic stages. Asparagine, proline, tryptophan and phenylalanine disappeared (Table 3) in the pre-autolytic period. Spots due to these amino acids were not seen on the chromatograms at zero day of autolysis. The maximum concentration, (2·454 g./100 g. dry mycelium) at the 9th day, diminished by 86% during the 9 days before autolysis. From the beginning of the autolytic phase to the end of the period studied the total concentration of the free amino acids decreased again by 81%, with respect to the concentration present at zero day of autolysis. Apart from serine, which was easily detectable throughout the whole cycle, no other amino acid was observed at the later stages of autolysis. Leucine, serine, threonine and alanine were the most abundant constituents in the pool; the sum of these four components formed more than 50% of the total content of amino acids present at each date.

DISCUSSION

Cessation of growth of *Aspergillus terreus* in these experiments seemed to take place when the carbohydrate source (glucose) had been almost completely consumed. The amount of glucose present in the culture filtrate (determined by Somogyi's method)

Table 3. *Intracellular free amino acids present in mycelium of Aspergillus terreus at various stages of growth*

	Time of incubation (days)										
	7	9	11	13	18	30	42	66	102	114	150
					Time of autolysis (days)						
					0	12	24	48	84	96	132
Amino acids	Amounts of amino acids (g./100 g. dry mycelium)										
Asparagine	—	*	*	—	—	—	—	—	—	—	—
Proline	*	*	*	—	—	—	—	—	—	—	—
Tryptophan	*	*	*	—	—	—	—	—	—	—	—
Phenylalanine	0·040	0·135	*	—	—	—	—	—	—	—	—
Unknown	0·034	0·030	0·018	0·012	0·010	*	—	—	—	—	—
Tyrosine	0·089	0·097	0·046	0·019	0·021	*	—	—	—	—	—
Aspartic acid	0·212	0·093	0·085	0·027	0·020	*	—	—	—	—	—
Norvaline	0·169	0·152	0·078	0·027	0·019	*	—	—	—	—	—
Lysine	0·072	0·302	0·220	0·064	0·016	*	*	*	—	—	—
Arginine	0·183	0·166	0·137	0·069	0·030	*	*	*	—	—	—
Leucine	0·189	0·344	0·147	0·065	0·046	*	*	*	—	—	—
Serine	0·281	0·352	0·261	0·102	0·072	*	*	*	*	*	*
Threonine	0·341	0·387	0·245	0·092	0·052	*	*	*	*	—	—
Alanine	0·382	0·396	0·212	0·075	0·052	*	*	*	*	—	—
Total amounts	1·995	2·454	1·454	0·552	0·388	0·288		0·162	0·060		

* Too small amount to be individually estimated; — absent.

stayed practically constant (Table 1) when autolysis set in. Crewther & Lennox (1953) found similar results for *A. oryzae* grown in a medium containing NH_4^+ as N source.

The decrease in mycelial dry weight by *Aspergillus terreus* (14·6%) in the present experiments is of the same order as that found by Ritter (1955) for *A. niger* (17·6%). A nearer value (14·4%) was reported by Ritter for a strain of *A. niger* which he called strain 'S'. The fungi in both cases were grown in an acid medium.

With respect to the behaviour of fat during autolysis it can be seen in Table 2 that a continuous decrease of mycelial lipids took place before and after the beginning of autolysis. Similar results were observed for *Aspergillus terreus* grown in a medium physiologically alkaline, in which there was a continuous diminution in the content of mycelial fat throughout the whole period of autolysis (Lahoz, 1967).

The increase in the concentration of free glucose in the mycelium of *Aspergillus terreus*, at a certain stage of autolysis, seems to be a common feature for both 'alkaline' (Lahoz, 1967) and 'acid' autolysis.

The disappearance of free amino acids from mycelium of *Aspergillus terreus* began at a stage in which autolysis, measured as decrease in mycelial weight, had not begun. Similarly the content of free amino acids in mycelium of *A. flavus* decreased during both the log and the autolytic phases (Pillai & Srinivasan, 1956), whereas for *Microsporum canis* the maximum concentration of the pool of amino acids was observed towards the end of the log phase, with a continuous decrease thereafter (Chattaway, Toothill & Barlow, 1962).

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Effect of Periodate on Competence in *Bacillus subtilis*

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SUMMARY

Treatment of competent cells of *Bacillus subtilis* with sodium periodate reduces markedly genetic transformation, at concentrations not significantly affecting cell viability. The action of periodate is not on DNA transforming activity. A reduction of transformation by this reagent is also obtained either if cells are treated before DNA addition or if they are first exposed to donor DNA for short time and then treated with periodate; if the cells have been pre-incubated with DNA for about 30 min. the subsequent addition of $NaIO_4$ is without effect. The action of periodate seems to be exerted on the adsorbtion and/or entry of DNA into the competent cells. The site of action of periodate is probably a component(s) of the cell wall involved in these processes.

INTRODUCTION

Bacterial cells which are susceptible to genetic transformation by DNA are said to be 'competent'. 'Competence' is a transitory state in the life cycle of the cell (Hotchkiss, 1954; Thomas, 1955) and different species of transformable bacteria require different growth conditions to reach this state. Conditions to render *Bacillus subtilis* cells competent were first worked out by Spizizen (1958); further studies were reported by Anagnostopoulos & Spizizen (1961), Young & Spizizen (1961), and by Nester (1964). Very little is known up to now about the biochemical and physiological events which determine the development of competence. The hypothesis that competence is determined by the synthesis of some enzyme or enzyme-like factor is supported by the findings of Pakula & Walczak (1963) in Streptococcus; of Tomasz & Hotchkiss (1964) in Pneumococcus, and of Charpak & Dedonder (1965) in *B. subtilis*; these authors have reported that the supernatant of a competent culture can confer this property on non-competent cells; the active factor present in the supernatant is believed to be a heat labile protein. Studies by Young & Spizizen (1963) in *B. subtilis* and by Ephrussi-Taylor & Freed (1964) in Pneumococcus suggest that competence could be due to the synthesis of an autolytic enzyme which would act upon the cell wall, forming a partial protoplast.

Due to the central role attributed to the cell wall for the development of competence, we have undertaken a study of the effect of sodium periodate on competent cells, prompted by the observation of Sneath & Lederberg (1961) that this reagent alters the surface of male cells of *Escherichia coli*, thus inhibiting bacterial conjugation.

The present paper will report the effect of periodate on competence in *Bacillus subtilis*. A preliminary account of this work has been published elsewhere (Polsinelli & Barlati, 1965). Inhibition of transformation in *Haemophilus influenzae* by periodate has been recently reported (Ranhand & Lichstein, 1966).

METHODS

Strains. The strains used are listed in Table 1.

Table 1. *List of Bacillus subtilis strains used*

Strain	Genotype	Origin
SB 25	*his*-2 *try*-2	J. Lederberg
SB 44	*try*-2	J. Lederberg
PB 3231	prototroph *str-r*	Derived from SB 25 in our laboratory

Symbols: *his* = histidine; *try* = tryptophan; *str-r* = streptomycin resistance.

Media. For massive cultures Penassay broth (Antibiotic Medium No. 3, Difco) was used. For the transformation assays the medium was that described by Spizizen (1958), herein referred to as S medium; for all platings Davis & Mingioli (1950) minimal medium (MM) was used.

DNA preparation. 400 ml. of culture of strain PB 3231, grown overnight in Penassay broth at 37° in an alternative shaker, were centrifuged; the cells washed twice with standard saline citrate (SSC: 0·15 M-sodium chloride and 0·015 sodium citrate), were resuspended in SSC (one fiftieth of the original volume) containing 1 mg. Bacto-lysozyme (Difco)/ml. and incubated at 37°; after 45 min., Duponol C (DuPont de Nemours, Wilmington, Del.) was added to a final concentration of 1 mg./ml. and the incubation continued for another 10 min.; then pronase (California Corp., Los Angeles, California), preincubated 1 hr at 37° in H_2O, was added to a final concentration of 1 mg./ml. and the lysate digested for another 4 hr at 37°. The lysate was then shaken with an equal volume of chloroform containing 4% isoamyl alcohol for 20 min., centrifuged 10 min. at 10,000 r.p.m., and the supernatant precipitated with 4 volumes of ethanol; the nucleic acid fibres, collected with a stirring rod and washed in 70% ethanol, were then dissolved in 10 ml. SSC and incubated for 30 min. at 37° with 50 μg. pancreatic ribonuclease/ml. (Fluka, Buchs SG, Switzerland) and 1 unit ribonuclease T_1 (Sankyo Co., Tokyo)/ml., both pre-heated for 10 min. at 80° in H_2O. The solution was then shaken once more with chloroform-isoamyl alcohol, centrifuged and the DNA was precipitated by adding to the supernatant 1/10 volume of acetate-EDTA (3 M-sodium acetate plus 0·001 M-EDTA, pH 7) and 0·54 volume of cold isopropyl alcohol (Marmur, 1961). The precipitated DNA was dissolved in 5 ml. SSC and the concentration was determined by the diphenylamine method (Dische, 1955) using deoxyadenosin-monophosphate as standard. RNA was determined by the orcinol method (Ashwell, 1957) using adenosin-monophosphate as standard.

For the preparation of ^{32}P-labelled DNA a 10 ml. culture of strain PB 3231, grown for 8 hr in MM medium supplemented with 0·1% Bacto-gelatin (Difco) was centrifuged and resuspended in the same volume of saline; 5 ml. of this suspension were added to 1000 ml. MM medium containing only 14 mg. KH_2PO_4 and 4 mg. K_2HPO_4, added with 1 mg. Bacto-gelatin and 5 mc carrier-free ^{32}P, as sodium phosphate (Sorin, Saluggia, Italy). After 15 hr of incubation at 37° in an alternative shaker the cells were used for DNA extraction following the procedure above described. The preparations of ^{32}P-labelled DNA used had a specific activity ranging from 8×10^4 to

10^5 per μg. and contained less than 5% RNA. Radioactivity of DNA was determined in a Packard Tri-carb scintillation Spectrometer.

Transformation techniques. Competent cells were prepared by the method described by Young & Spizizen (1961), slightly modified. Incubation with DNA was performed at 34°. A concentration of 2 μg./ml. of donor DNA was used, unless otherwise stated. Exposure to DNA was stopped by adding final concentrations of 20 μg. deoxyribonuclease (Worthington Biochemical Corp., Freehold, N.J.)/ml. and 10^{-3} M-magnesium chloride and the colonies transformed to tryptophan independence were scored following selection on MM medium supplemented with 20 μg. histidine/ml. Appropriate controls for backmutation were always run.

Periodate treatment. Competent cells to be treated with sodium periodate were centrifuged at 5000 rev./min. for 10 min. at room temperature, washed once with same volume of the salt solution of Spizizen's medium lacking sodium citrate and resuspended in saline, at a concentration of approximately 10^9 cells/ml. Unless otherwise stated, to one volume of cell suspension, four volumes of fresh solution of sodium periodate of different concentrations were added and the mixture incubated for 10 min. at 37° with agitation. The action of periodate was arrested by adding glucose to a final concentration of 1% (w/v); the suspension was centrifuged as above and the cells resuspended in S medium (about 5×10^8 cells per ml.) were finally tested for transformation.

^{32}P-DNA uptake. Competetent cells were exposed to ^{32}P-DNA (0·1 μg./ml.) at 34° for different times; the reaction was arrested by addition of deoxyribonuclease (DNAse) and magnesium chloride, followed by incubation for 5 min. at 37°. The cultures were then diluted three-fold with S medium, centrifuged and resuspended in the same medium; a portion was used for transformation tests and the remainder was filtered through a membrane filter (Millipore Corp., Malakoff, France), washed 5 times with 10 ml. samples of *S* medium, and counted for radioactivity.

RESULTS

Effect of periodate on viability and transformation

Strain SB 25 competent cells were treated for 10 min. with different concentrations of sodium periodate, according to the procedure described in Methods and incubated immediately afterwards with 2 μg./ml. of strain PB 3231 DNA for 30 min. The treated cells were then tested for transformation to tryptophan independence and for survival. Figure 1 shows that both viability and transformation were reduced by increasing concentrations of periodate. However, periodate concentrations up to 5×10^{-4} M had no significant effect on the viability, while markedly reducing transformation.

Action of periodate on DNA transforming activity

The effect of periodate could be ascribed either to action on the cells or to a direct damage on DNA; to choose between these possibilities two experiments were carried out.

DNA was dissolved in saline at a concentration of 20 μg./ml. and incubated for 10 min. at 37° with different concentrations of periodate; addition of glucose, to a final concentration of 1%, stopped the action of the reagent. A fraction 0·1 ml. of this solution was tested for transforming activity. Table 2 shows that periodate did

not appreciably affect the biological activity of DNA, thus ruling out the second possibility. To check whether the effect was not due to the production of some other toxic substance through the interaction of periodate with cells and glucose, in another experiment, to 1 ml. of a culture of competent cells treated with 5×10^{-4} M-periodate, 2 μg. DNA/ml. and an equal amount of untreated competent cells were added. After 30 min. of incubation at 34°, samples were plated to measure the frequency of transformation for tryptophan prototrophy. Results reported in Table 3 show that the transforming activity of DNA in those conditions was not affected.

Table 2. *Transforming activity of DNA after treatment with different concentrations of periodate for 10 min. at 37°*

$NaIO_4$ concentration	Transformation frequency to tryptophan prototrophy*
No periodate	$1{\cdot}2 \times 10^{-3}$
$0{\cdot}05 \times 10^{-3}$M	$1{\cdot}1 \times 10^{-3}$
$0{\cdot}10 \times 10^{-3}$M	$1{\cdot}4 \times 10^{-3}$
$1{\cdot}00 \times 10^{-3}$M	$1{\cdot}1 \times 10^{-3}$

* Donor strain, PB 3231 prototroph; recipient strain, SB 25 *his*-2 *try*-2.

Table 3. *Biological activity of DNA incubated with competent cells treated with 5×10^{-4} M-periodate for 10 min.*

	Experiment number				
	1	2	3	4	5
Reagents in order of addition	Competent cells — Glucose —	Competent cells $NaIO_4$ Glucose —	Competent cells — Glucose DNA	Competent cells $NaIO_4$ Glucose DNA	Competent cells $NaIO_4$ Glucose DNA + competent cells
Frequency of tryptophan independent colonies	$6{\cdot}6 \times 10^{-9}$	$8{\cdot}3 \times 10^{-9}$	$2{\cdot}1 \times 10^{-4}$	$1{\cdot}3 \times 10^{-5}$	$2{\cdot}7 \times 10^{-4}$

Exposure of competent cells first to DNA and then to periodate

The two experiments described above demonstrate that the phenomenon under study must be due to the reaction of periodate with the cells; such action can be exerted either on the process of binding and incorporation of DNA into the cells, or on the subsequent intracellular events leading to the integration of the marker in the recipient DNA. It is also possible that the effects on cells could be due to inactivation of one or more nuclei in multinucleate cells, so that there could be a decrease of transformability but not of the viability at low concentrations of periodate. If the action is in the process of binding and incorporation of DNA there is a way to decide between this and the other two explanations. Since it has been shown that, before entering into the competent cell, the DNA bound to its surface remains sensitive to deoxyribonuclease (Lerman & Tolmach, 1957; Levine & Strauss, 1965), the following experiments were performed in order to determine whether periodate acts in this extracellular stage, rather than in the following intracellular one.

To a series of 250 ml. flasks containing 10 ml. of ice-cold competent cells, DNA was added to a final concentration of 2 μg./ml. and the flasks incubated at 34°. At different time intervals the content of each flask was poured in 3 volumes of ice-cold S medium, filtered through a Millipore membrane and washed 5 times with 10 ml. of S medium salts lacking sodium citrate. In the case that we call 'zero time' ice-cold cells were diluted and washed immediately after the DNA addition. Cells were then resuspended from the filter membrane with 1 ml. of saline and 0·3 ml. samples were distributed into three test tubes; to one tube 0·7 ml. of 2×10^{-4} M-periodate was added; to another one 0·7 ml. of deoxyribonuclease solution; to the third one 0·7 ml. of water. After 10 min. of incubation at 37°, 2 ml. of S medium containing 1% glucose were added and the cells were tested for transformation. The

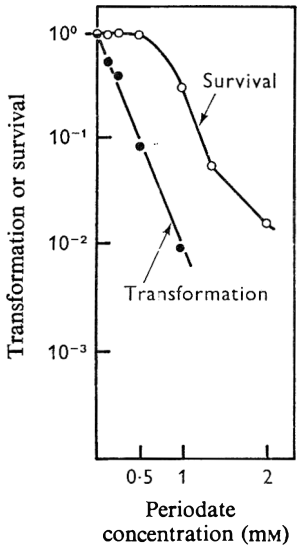

Fig. 1

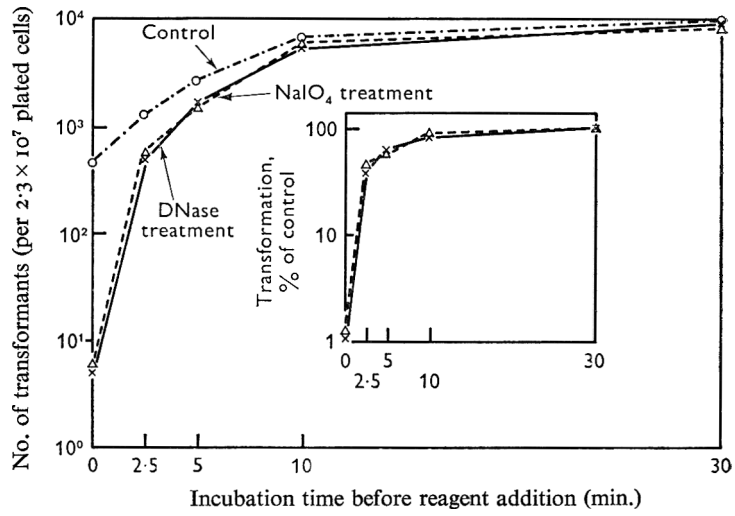

Fig. 2

Fig. 1. Survival and transformation of competent cells incubated at 37° for 10 min. with different concentrations of sodium periodate. Transformation frequency in the cells not exposed to periodate was $0{\cdot}6 \times 10^{-3}$.

Fig. 2. Transformation in cell cultures of SB 25 exposed to DNA (2 μg./ml.) for different times and then treated with deoxyribonuclease or periodate or water. The insert reports the same values as percent of the control.

data are reported in Fig. 2. The curve indicated as 'control' refers to transformation obtained with cells which were exposed only to DNA and the water. The transformation data from cultures which, after DNA incubation, were also treated with periodate or DNase are reported in the other two curves. The graph shows that the cultures incubated with DNA for 30 min. before adding periodate or DNAse or water gave similar amount of transformation. On the other hand, transformation obtained in tubes which were treated with periodate or with DNAse after a short time of incubation with DNA, was strongly reduced as compared to the transformation in the untreated tubes. Periodate seems therefore to act on the same stage in which DNAse acts. At 'zero time' the control tube shows appreciable transformation; this

is due to the rapid attachment of DNA to the cell that must be so strong as to be insensitive to immediate dilution.

Reduction of DNA uptake by periodate

Experiments were performed in order to test directly if the reduction of transformation by periodate could be attributed to reduction of DNA uptake by the competent cell. Incorporation of DNA into the cells was followed on strains SB 25 and SB 44, the latter, a non-transformable strain, serving as a control for a specific incorporation by non-competent cells. Five hundred ml. of cell culture of each strain

Table 4. *^{32}P-DNA uptake and transformation in the competent strain SB 25. Cells not submitted (a) or submitted (b) to periodate action*

Time of incubation with DNA* (0·1 μg./ml.) (min.)	(a) Cells not treated with periodate		(b) Cells treated with 2×10^{-4} M-periodate/10 min. at 37°	
	^{32}P-DNA uptake (c.p.m./ $5{\cdot}7 \times 10^9$ cells)	No. transformants (per $5{\cdot}7 \times 10^7$ plated cells)	^{32}P-DNA uptake (c.p.m./ $5{\cdot}7 \times 10^9$ cells)	No. transformants (per $5{\cdot}7 \times 10^7$ plated cells)
0	86	$< 1 \times 10^{-8}$	123	$< 1 \times 10^{-8}$
5	653	1215	140	46
15	1570	3025	172	108
30	2953	5302	213	201

* Specific activity of DNA: 10^6 c.p.m./μg.

Table 5. *^{32}P-DNA uptake and transformation in the non-competent strain SB 44. Cells not submitted (a) or submitted (b) to periodate action*

Time of incubation with DNA* (0·1 μg./ml.) (min.)	(a) Cells not treated with periodate		(b) Cells treated with 2×10^{-4} M-periodate/10 min. at 37°	
	^{32}P-DNA uptake (c.p.m./ $5{\cdot}7 \times 10^9$ cells)	No. transformants	^{32}P-DNA uptake (c.p.m./ $5{\cdot}7 \times 10^9$ cells)	No. transformants
0	82	$< 1 \times 10^{-8}$	63	$< 1 \times 10^{-8}$
5	90		53	
15	57		76	
30	65		67	

* Specific activity of DNA: 10^6 c.p.m./μg.

were centrifuged, washed with S medium lacking sodium citrate, resuspended in 8 ml. of saline and divided in two portions; to one portion 12 ml. of 2×10^{-4} M-periodate were added, to the other 12 ml. of water. After 10 min. at 37°, the cells of each portion were resuspended in 40 ml. of transformation medium, distributed in four 100 ml. flasks (10 ml. per flask), and incubated with 0·1 μg. ^{32}P-DNA/ml. After different time intervals the cultures were treated with DNAse, tested for transformation and counted for radioactivity as described in Methods. In the 'zero time' flask the 10 ml. culture was first diluted three fold with S medium and then treated with DNA

preincubated with DNase for 5 min. at 37°. The data are reported in Tables 4 and 5. The experiments with the competent strain SB 25 shows: (*a*) that both DNA uptake and number of transformants increase with the incubation time; (*b*) that the uptake of DNA and the transformation are both significantly reduced in the cells treated with periodate as compared to the untreated ones. On the other hand, the non-competent strain SB 44 gave no detectable transformation, as expected, and a low DNA uptake, which remained roughly constant during the incubation time. No significant differences in the uptake were also found in this strain between cells treated and not treated with periodate. On the assumption that also the cells of the competent strain can take up a similar fraction of DNA by some specific mechanism

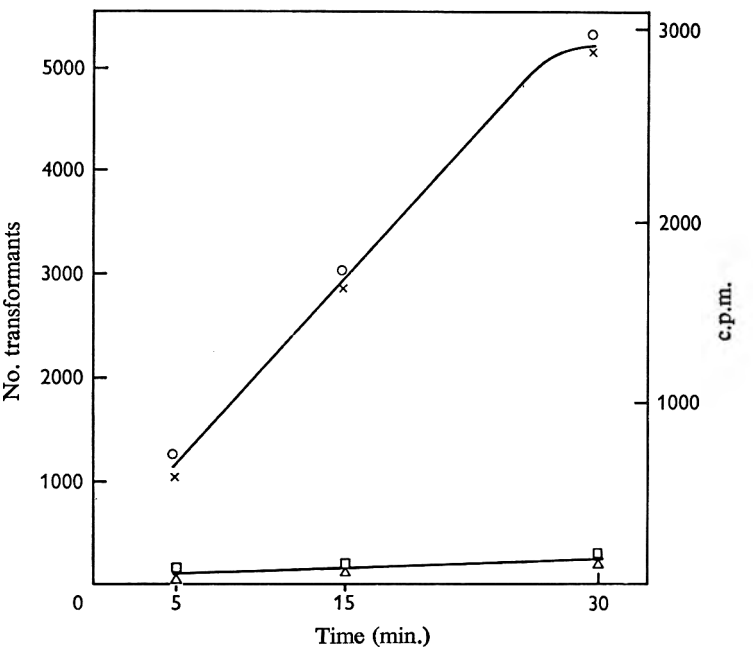

Fig. 3. DNA uptake and transformation in competent cells of SB 25 treated or not treated with periodate before the exposure to donor DNA. Data plotted have been corrected for the uptake found in non competent cells of SB 44. Transformation (O——O) and radioactivity (×——×) in cells not treated with periodate. Transformation (△——△) and radioactivity (□——□) in cells treated with periodate.

not involved in the transformation process, the counts found in the non-competent cells have been subtracted from those found in the competent ones; the data so elaborated are plotted in Fig. 3. In the same figure also the transformation data have been reported, and the scales on the ordinate have been arbitrarily set at a ratio that underlines the striking parallelism between the two phenomena.

DISCUSSION

The results reported above show that treatment with periodate reduces the transformation of competent cells of *Bacillus subtilis* at concentrations not appreciably affecting cell viability. While the dependence of inactivation of transformation on

periodate concentration indicated a 'single hit' process, cellular death appeared to be caused by a more complex mechanism. The possibility that periodate could affect transformation by acting in some way on transforming DNA was ruled out by the experiments in Table 2. The possibility that the donor DNA could be affected by some compound formed during cell periodate treatment, and still present in cell culture incubated with DNA, was also eliminated by the data in Table 3. It is also unlikely that, once the donor DNA has entered the competent cell, periodate may affect the transformation process, since competent cells first exposed to DNA for 30 min. and then treated with periodate were transformed as well as the controls (Fig. 2). This experiment suggests also that the effect of periodate may be due to an alteration of some factor(s) involved in the maintenance of competence and not to a generalized damage on the cells; otherwise, one would expect reduction of transformation also in cells treated with periodate after 30 min. of DNA incubation. On the other hand, for shorter incubations, periodate affected the transformation in a way analogous to DNase. The reduction by DNase, as mentioned above, is due to the fact that DNA, after binding to the competent cells, is still sensitive for a certain length of time to the enzyme (Lerman & Tolmach, 1957; Fox & Hotchkiss, 1960; Levine & Strauss, 1965). The reduction determined by periodate could therefore be attributed either to its interference in the process of entering of the DNA already bound to the cell or to the destruction of the receptor to which it is bound.

The hypothesis that periodate inhibits transformation by reducing DNA incorporation into competent cells is supported by the data in Table 4, which show that both DNA uptake and transformation were significantly reduced by periodate treatment. A certain amount of radioactivity was also incorporated by non-competent cells (Table 5) in absence of transformation, as already observed in *B. subtilis* by Young & Spizizen (1963). Such radioactivity did not increase with time of exposure to DNA and was not reduced when cells were treated with periodate. The data plotted in Fig. 3, which have been corrected for the aspecific uptake found in the non-competent strain, show a good parallelism between the time dependence of DNA incorporation and transformation; this parallelism is even more marked if one observes the effect of periodate on the two phenomena, that seem to be affected in a similar way. The experiment demonstrates therefore that the treatment with periodate reduces the ability of the cell to take DNA up, and that the uptake so determined is a good measure of—or at least parallel with—the process leading to bacterial transformation, in agreement with the results of Lerman & Tolmach (1957), Goodgal & Herriott (1957), Fox (1957), Young & Spizizen (1961 and 1963). The site of action of periodate is probably a component(s) of the cell wall, involved in the binding and/or in the permeation of transforming DNA.

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Inducible β-lactamase in Enterobacter

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SUMMARY

Production of β-lactamase by nine strains of the genus *Enterobacter* (eight *Enterobacter cloacae*, one *E. aerogenes*) was studied to determine its inducibility. Induction was observed with benzylpenicillin (500 μg./ml.) in all except one strain. Cultures were examined to locate the enzyme; it was found that in exponential growth the enzyme was cell-bound, and in stationary phase cultures much of it was in the culture medium. Maximum enzyme activity was only demonstrated after cell-breakage. Substrate profiles of crude enzyme preparations were examined and it was observed that the enzymes were 20–80 times more active against cephalosporins than against benzylpenicillin. Evidence is presented which suggests that one strain of *E. cloacae* produced two β-lactamases, an inducible cephalosporinase and a constitutive penicillinase.

INTRODUCTION

Abraham & Chain (1940) were the first to describe the production of penicillinase (β-lactamase) by *Escherichia coli* and since then there have been many reports of the synthesis of this type of enzyme by other Gram-negative species (see Citri & Pollock, 1966). Unlike the β-lactamases from Gram-positive species, however, these enzymes are not usually inducible (Smith & Hamilton-Miller, 1963; Citri & Pollock, 1966) and where induction has been shown to occur, it is of a low order (Hamilton-Miller, 1963; Ayliffe, 1964, 1965; Citri & Pollock, 1966). *Pseudomonas aeruginosa* has, in contrast, been shown to produce an inducible β-lactamase, but only in the presence of high concentrations of inducer (Sabath, Jago & Abraham, 1965). The term β-lactamase includes two types of enzyme—penicillinases and cephalosporinases—which frequently show overlapping specificities. Among Gram-negative bacteria, *Klebsiella aerogenes* and *Proteus mirabilis* produce penicillinases (Ayliffe, 1965), while members of the genera *Enterobacter* (*Aerobacter*), *Serratia*, *Hafnia*, and *Proteus morgani* and *Pseudomonas aeruginosa* produce cephalosporinases (Fleming, Goldner & Glass, 1963; Ayliffe, 1965; Sabath *et al.* 1965). Furthermore, many species of Enterobacteriaceae can become infected with R-factors and some, at least, of these carry genes responsible for synthesis of penicillinase (Datta & Kontomichalou, 1965). The present paper describes the production of one or more types of β-lactamase by nine Enterobacter strains—either *Enterobacter cloacae* or *E. aerogenes*—and reports an investigation of the kinetics of enzyme production to see whether it is inducible in any of these strains. Whereas Smith & Hamilton-Miller (1963) studied six Enterobacter (Aerobacter) strains and found them all to produce constitutive penicillinase, Fleming and his collaborators showed that a strain of *E. cloacae* produced a high concentration of

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cephalosporinase, presumably constitutively (Fleming *et al.* 1963). All the strains of Enterobacter examined in the present work produced cephalosporinase and in eight of these production was inducible. Enzyme synthesis was increased at least 50-fold in four strains. Examination of the substrate profile of one of the inducible strains suggests that, in addition to inducible cephalosporinase, it produced another β-lactamase constitutively.

METHODS

Bacterial strains and identification. Enterobacter cloacae strains 214, 249, 251, 252, 255, 256 and 257 were isolated in 1964 and 1965 from patients at Hammersmith Hospital. With the exception of strains 251 and 252, which were conceivably different isolates of the same strain, the organisms were unrelated to each other. *Enterobacter cloacae* P99 was kindly supplied by Dr Cynthia O'Callaghan (Glaxo Laboratories) and *E. aerogenes* 229 by Miss Pamela Waterworth (Department of Bacteriology, Royal Postgraduate Medical School).

Species were identified according to the scheme of Cowan & Steel (1965), whose basis for distinction between the genera *Enterobacter* and *Klebsiella* included two criteria: (*a*) motility—enterobacters are motile and klebsiellas are non-motile; (*b*) production of ornithine decarboxylase—enterobacters produce this enzyme and klebsiellas do not.

Antibiotic resistance. Minimum inhibitory concentrations (m.i.c.) of benzylpenicillin, ampicillin and cephaloridine were determined by using two-fold dilutions in nutrient agar with two inoculum sizes (about 10^6 and 10^3 organisms). All the *Enterobacter cloacae* strains were resistant to these drugs at 1000 μg./ml., regardless of the size of the inoculum. *Enterobacter aerogenes* strain 229 was 10–20 times less resistant than the *E. cloacae* strains.

Resistance patterns were examined by using Multodisks (Oxoid Ltd.) containing the following compounds: chloramphenicol (Cm) 50 μg.; colomycin (Col) 200 μg.; nitrofurantoin (Nitro) 200 μg.; sulphafurazole (Sul) 500 μg.; ampicillin (Amp) 25 μg.; kanamycin (Kan) 30 μg.; streptomycin (Sm) 25 μg.; tetracycline (Tet) 50 μg.; The resistance patterns were: strain 229, Amp, Sul; 249, Amp, Tet, Sul, Sm; P99, Amp, Tet, Sul, Sm, Cm; 214, 251, 252, 255, 256 and 257, Amp, Tet, Sul, Sm, Nitro.

There was no evidence that resistance to the two penicillins and cephaloridine could be transferred to *Escherichia coli* K12. This was tested by growing each strain overnight in mixed culture with *E. coli* K12 and heavily inoculating the mixture on a defined medium containing appropriate antibiotic and dulcitol as sole carbon source. Enterobacters could not, whereas *E. coli* K12 could, utilise dulcitol and grow on this medium. Conjugation and genetic transfer could be demonstrated by the method used, for resistance to chloramphenicol and tetracycline was transferred from strain P99. Moreover, an R-factor carrying a determinant for resistance to chloramphenicol was introduced by conjugation into strain 214, and the resulting culture could transfer chloramphenicol resistance to *E. coli* K12, but no mobilization of genes mediating β-lactamase production was demonstrated.

Media. Oxoid Nutrient Broth No. 2 was used for all fluid cultures. The medium was solidified when required by adding 1·5% (w/v) Oxoid agar.

Penicillins and cephalosporins. Commercial preparations of the following were used: benzylpenicillin and cephaloridine (Glaxo Laboratories Ltd., Greenford, Middlesex)

and ampicillin (Beecham Research Laboratories, Brockham Park, Betchworth, Surrey). Benzylpenicillin will be referred to as penicillin. Other compounds were received as gifts from the manufacturers: methicillin, cloxacillin, phenethicillin, 6-aminopenicillanic acid from Beecham Research Laboratories; quinacillin from Boots Pure Drug Company, Nottingham; phenoxymethylpenicillin from Eli Lilly and Company, Indianapolis, Ind., U.S.A.; cephalothin, cephalosporin C, 7-phenylacetamidocephalosporanic acid, cephaloridine from Glaxo Laboratories, Stoke Poges, Bucks.

Growth experiments. The bacteria were grown in conical flasks shaken in a water-bath at 37°. The volume of culture was one fifth of the flask capacity. Extinctions were measured with a Unicam SP 600 spectrophotometer at wavelength 675 mμ. Conversion of extinction to bacterial dry weights was made by reference to a standard curve prepared for *Enterobacter cloacae* strain 214. Exponential cultures referred to in the text were cultures grown as above and harvested after 3 hr.

Induction of β-lactamase. In experiments to determine the inducibility of β-lactamase, an overnight culture was diluted 20-fold into fresh medium and grown with shaking at 37°. After 1 hr, inducer was added and shaking continued for a further 2 hr before the culture was harvested.

Where a large yield of enzyme was required, as in the study of substrate profiles, overnight cultures were used. In some, inducer was added to obtain sufficient enzyme for the purpose. Two additions of penicillin were made to 600 ml. culture at 1 and 4 hr after subculture (300 mg. at each addition) because penicillin itself was hydrolysed and it was thought likely that greater induction would result if the inducer was replenished. After the second addition of penicillin, growth was continued for 16–18 hr.

Harvesting procedure. Normally, cultures were centrifuged at 5000 *g* at 4° for 20 min, the deposited organisms washed once with 0·01 M-phosphate buffer (pH 7·0) and resuspended in buffer, giving a final bacterial concentration equivalent to 5–15 mg. dry wt/ml. When a preparation was required for substrate specificity experiments, a concentration equivalent to 20–40 mg. dry wt/ml. was used.

Breakage of organisms. Organisms were disrupted in 5 ml. volumes of suspension for 4 min. with a Mullard ultrasonic disintegrator. Excessive heating was prevented by immersing the preparation in a bath of flake ice.

Substrate specificity experiments. Suspensions of broken organisms were centrifuged at 40,000 *g* at 4° for 60 min. and the supernatant fluid used for determining the relative rates of hydrolysis of different substrates.

Induction experiments. Suspensions of broken organisms were used without further treatment (i.e. without high speed centrifugation).

Enzyme assays. The iodometric method of Perret (1954) was used but when cephalosporin hydrolysis was measured, the calculation was based on the observation of Alicino (1961) that 1 mole of cephalosporin C hydrolysed is equivalent to 4 equivalents of iodine and not approximately 8 equivalents as with penicillins. Enzyme activity was assayed in culture fluids, whole culture samples, concentrated suspensions of bacteria, preparations of broken bacteria, or supernatant fluid from these preparations. Corrections were made for non-specific uptake of iodine by substrates and by bacterial extracts by testing controls in which enzyme preparation was added to substrate after the addition of iodine reagent. Enzyme activities are expressed in units similar to those defined by Pollock & Torriani (1953) but at pH 6·0. Substrate concentration was 0·007 M in 0·2 M-phosphate buffer (pH 6·0).

RESULTS

β-lactamase

The absolute activities of enzyme produced by log-phase organisms which had been disrupted are shown in Table 1. Two strains (P99, 214) were distinctly more active in hydrolysing cephaloridine than the remaining strains. Strain 249 alone catalysed the hydrolysis of ampicillin and the difference between penicillin and cephaloridine hydrolysis was less with this strain than with the others, which predominantly produced cephalosporinase.

Table 1. *Absolute activities of β-lactamase produced by 9 Enterobacter strains*

	Substrate		
	Penicillin	Ampicillin	Cephaloridine
Strain	β-lactamase (units/mg. dry wt bacteria)		
P99	24	–	1380
214	3	–	200
249	19	28	32
251	–*	–	3
252	–	–	2·3
255	–	–	1·6
256	–	–	1·8
257	–	–	49
229	–	–	6

*– = less than 0·3 units/mg. dry wt

Location of enzymes

Measured volumes of cultures in the exponential or in the stationary phase (3 or 16–18 hr, respectively) were used to locate β-lactamase activity. Total activities were calculated for whole cultures, supernatant fluids, intact and broken organisms. The distribution of enzyme activity is shown in Table 2. Also shown is the 'cryptic ity factor' which is the ratio of activities in broken : intact organisms. In log-phase cultures the proportion of enzyme in the supernatant fluid was less than 12% of the total activity (supernatant fluid activity + activity in broken organisms) whereas in stationary-phase cultures the supernatant fluid contained 40–60%. Addition of penicillin did not increase the amount of extracellular enzyme. In most cases maximum enzyme activity was only evident after breaking the organism.

Induction

To test whether penicillin would induce β-lactamase in *Enterobacter cloacae*, strain 256 was grown in the presence of a wide range of concentrations (10 μg.–12 mg./ml.). The activity of broken organisms was measured against penicillin, ampicillin and cephaloridine; ampicillin was not hydrolysed and therefore is not recorded (Table 3).

Maximal induction was observed with penicillin, 500 μg./ml., and hydrolysis of cephaloridine was increased 117 times with this concentration. Although concentrated suspensions of bacteria were tested, the basal rate of hydrolysis of penicillin, in the absence of inducer, was so low that it was not possible to assess the induction ratio for penicillinase activity. In the presence of inducer, however, the increased rate of hy-

drolysis of penicillin was comparable with that of cephaloridine and was consistent with the view that both penicillinase and cephalosporinase activities were manifestations of the same enzyme. Induction took place even when penicillin concentration (6 mg./ml.) caused lysis. In all subsequent induction experiments the inducer concentration was 500 μg./ml.

Table 2. *Distribution of β-lactamase activities in cultures*

			'Cephalosporinase'			'Penicillinase'		
	Time of harvest (hr)	Strain no.	Total activity (units)*	% total activity in sf.†	'Cryptic factor'§	Total activity (units)*	% total activity in sf.†	'Cryptic factor'§
Untreated	3	P99	9[illegible],300	6	2·1	1,300	—¶	3·3
	3	214	1[illegible],200	5	2·3	190	—	1·4
	3	249	3,055	3	1·1	2,247	3	9·9
	3	257	1,610	11	1·0	< 18	—	—
	16–18	257	25,850	66	1·0	nt	nt	nt
+Penicillin (500 μg./ml.)	3	P99	209,500	3	2·8	2,500	2	3·2
	3	214	118,200	2	6·5	1,640	4	3·9
	3	256	21,800	2	1·1	360	—	2·0
	16–18	256	13,800	40	1·4	220	41	1·5
	16–18	252	68,200	44	1·6	940	48	1·0
	16–18	255	82,400	27	1·8	nt	nt	nt
	16–18	257	127,100	64	1·9	nt	nt	nt

The substrates tested were cephaloridine and penicillin.
* Total activity = activity in broken cells + activity in culture supernatant.
† sf = supernatant fluid.
§ Ratio of disrupted: intact cell activities.
¶ = Activity too low for measurement.
nt = not tested.

Table 3. *Induction of β-lactamase in Enterobacter cloacae strain 256*

Inducer	Concentration (μg./ml.)	'Penicillinase'* units/mg. dry wt	'Cephalosporinase'* units/mg. dry wt	'Cephalosporinase'* Induction ratio†
None	—	< 0·3	2	—
Penicillin	10	< 0·3	6	3
	100	0·5	25	12·5
	500	3	234	117
	1,000	2	121	60·5
	6,000	< 0·3	16	8
	12,000	< 0·3	< 2	—
Ampicillin	500	1	92	46
Cephaloridine	500	3	198	99
Methicillin	500	< 0·3	0·4	0·2

* Hydrolysis of penicillin and cephaloridine respectively.
† Ratio of activities induced:uninduced preparations.

Ampicillin and cephaloridine also induced enzyme production in strain 256 but methicillin inhibited cephaloridine hydrolysis (see Table 3).

The results of induction experiments with the remaining 8 strains are recorded in Table 4, included in which are the relevant figures of strain 256 for comparison. Hydrolysis of ampicillin is omitted since only one strain (*Enterobacter cloacae* 249)

hydrolysed this substrate. Strain P99 was the only one which showed no induction. When strain 249 was grown in the presence of penicillin, the rate of hydrolysis of cephaloridine increased compared with the uninduced control but there was no change in the rates of hydrolysis of either ampicillin or penicillin. In all other strains, increase in penicillinase and cephalosporinase activities paralleled each other.

The effect of methicillin on cultures of P99 and 214 was not tested for the following reason. There was no detectable hydrolysis of methicillin by crude enzyme preparations and, further, incubation of these preparations with methicillin (2 mM final concentration) for 15 min. completely abolished activity. The nature of this inhibition has not been determined. From Table 4, however, it will be seen that methicillin inhibited β-lactamase of strains 229 and 256, had no effect on 249 or 255, but induced 251, 252 and 257. Thus, 8 of 9 strains examined produced β-lactamase inducibly.

Table 4. *Induction of β-lactamase in 9 Enterobacter strains. Hydrolytic activities in units/mg. dry wt of bacteria*

	Uninduced		Induced			
	Substrate		Penicillin (500 μg./ml.) Substrate		Methicillin (500 μg./ml.) Substrate	
Strain	Penicillin*	Cephaloridine*	Penicillin	Cephaloridine	Penicillin	Cephaloridine
P99	24	1300	26 (1·0)†	1325 (1·0)	nt	nt
214	3	200	16 (5·3)	945 (4·7)	nt	nt
251	< 0·2	3	6	328 (109)	1	78 (26)
252	< 0·2	2	3	238 (119)	2	132 (66)
255	< 0·2	2	1	104 (52)	< 0·2	2 (1·0)
256	< 0·3	2	3	234 (117)	< 0·3	0·4 (0·2)
257	0·4	28	4 (10)	249 (9·7)	1 (2·5)	71 (2·5)
249	20	28	21 (1·0)	100 (3·6)	21 (1·0)	30 (1·0)
229	0·25	6	2 (8)	49 (8·2)	< 0·1	< 0·06

* Hydrolysis of penicillin and cephaloridine respectively; measured in preparations from concentrated suspensions of organisms

† ( ) Ratio of induced:uninduced activities.

nt = not tested.

Relative activities of crude enzyme preparations

Crude enzyme preparations from induced and uninduced cultures were examined for their relative activity against different penicillins and cephalosporins (Table 5). Methicillin, cloxacillin and quinacillin were also tested; they were resistant to hydrolysis. Strains P99, 214, 251, 252, 255, 256 and 257 (all *Enterobacter cloacae*) had broadly similar profiles but strain 257 may have been significantly different with respect to its action on cephalosporin C. Strain 229 (*E. aerogenes*) was not as active against either cephalosporin C or cephaloridine as were the above 7 strains. Hydrolysis of ampicillin, phenoxymethylpenicillin, phenethicillin and 6-aminopenicillanic acid by strain 249 did not change significantly when an induced preparation was tested, but destruction of the cephalosporins did increase. It seems likely therefore that this strain produced more than one β-lactamase.

DISCUSSION

One of the difficulties in comparing the work described here with earlier studies on β-lactamase in Gram-negative bacteria stems from taxonomic confusion. In the present work the classification of Cowan & Steel (1965) has been used. That members of the

Table 5. *Relative activities of Enterobacter crude enzyme preparations from uninduced and induced (IND) cultures*

Substrate	Strains											
	P99	214	251	251 (IND)	252 (IND)	255 (IND)	256 (IND)	257 (IND)	229	229 (IND)	249	249 (IND)
Penicillin	100	100	100	100	100	100	100	100	100	100	100	100
Ampicillin	–*	–	–	–	–	–	–	–	–	–	147	150
Phenoxymethyl-penicillin	79	69	–	68	90	87	79	80	–	50	56	50
Phenethicillin	–	–	–	–	–	–	–	–	–	–	24	30
6-Aminopenicillanic acid	–	–	–	–	–	–	–	–	–	–	176	160
Cephaloridine	7400	8030	6560	6550	8750	8000	7400	7000	4200	2420	133	800
Cephalothin	1180	2640	2360	1990	2700	2370	2400	2130	2340	2560	15	260
Cephalosporin C	6550	6180	6560	5950	7120	7610	6660	3970	3775	1900	14	680
7-Phenylacetamido-cephalosporanic acid	1730	2220	–	1800	2200	2120	2290	1965	2680	2130	11	185
Absolute rate of hydrolysis of penicillin (units/mg. dry wt)	20	8·6	0·014†	2·6	0·6	1·1	1·3	2·3	0·03†	2·2	10·7	12

*– Activity too low for measurement.
† These values were calculated from the induction ratio observed with cephaloridine as substrate.

tribe Klebsiellae have been called by a variety of names may well account for the fact that the strains reported here synthesized cephalosporinase while strains identified as members of the genus *Aerobacter* (*Enterobacter*) by Hamilton-Miller, Smith & Knox (1965) synthesized penicillinase. The enzymes described in the present paper are all cell-bound, at least in exponential growth. Breakage of the organisms liberated some β-lactamase; the fact that 40–60% of the enzyme was liberated into the culture medium of overnight cultures may reflect lysis of stationary-phase organisms. Because cell damage readily causes liberation of enzyme, penicillin in high concentrations might be expected to have this effect by making the organisms rather more leaky. This argument has been used by Sabath *et al.* (1965) to explain the presence of large quantities of enzyme in culture fluids of *Pseudomonas aeruginosa* grown exponentially in the presence of 10 mg. penicillin/ml. In contrast, no such enzyme liberation was detected with the Enterobacter cultures examined here, even after treatment with 500 μg. penicillin/ml.

Comparison of the substrate profiles of crude enzyme preparations from the 9 Enterobacter strains showed two main types of pattern. Strains P99, 214, 251, 252, 255, 256, 257 (all *E. cloacae*), and perhaps 229 (*E. aerogenes*) showed broadly similar cephalosporinase activities which could well represent the same, or very closely related, enzymes and which might be characteristic of the genus. Strain 249 (*E. cloacae*), on the other hand, was decidedly different and had a broader spectrum of activity. Of the 9 strains examined, the enzymes of strains 251, 252, 255 and 256 were highly inducible by penicillin and had induction ratios greater than 50 (Table 4). With the exception of strain 249, cephalosporinase and penicillinase activities were likely to be manifestations of the same enzyme. Cephalosporinase activity of strain 249 was increased approximately four-fold in the presence of penicillin and there was some evidence that the strain produced two β-lactamases, inducible and constitutive. The induction ratio for this strain cannot be accurately calculated since it is not possible to assess the relative contribution of each enzyme in any given preparation.

The substrate profiles reported here are different from that reported by Datta & Richmond (1965) for *Escherichia coli* carrying an R-factor, indeed there is no evidence that these enterobacters carry R-factors controlling β-lactamase synthesis. The β-lactamase produced by the *Pseudomonas aeruginosa* strain used by Sabath, *et al.* (1965) was similar to those described here in that it was relatively more active against cephalosporins, but it differed in being active against ampicillin. Two Proteus strains examined by Hamilton-Miller *et al.* (1965) appeared to make enzymes which bore some resemblance to Enterobacter enzymes. *Enterobacter cloacae* P99 came indirectly from Dr P. C. Fleming in Toronto and was the same strain about which he and his colleagues reported (Fleming *et al.* 1963).

No studies in the genera *Enterobacter*, *Proteus* or *Pseudomonas* have located genes for β-lactamase production at specific chromosomal sites; it remains possible therefore that all these genes are extrachromosomal, but the close similarity of β-lactamases of *E. cloacae* strains described here, however, suggests that the enzyme was species-specific.

The absence of highly inducible β-lactamase from strains of Enterobacteriaceae may reflect on the methods used for investigating this property. Hitherto inducer concentrations have been low as compared with what appears to be optimal for *Pseudomonas aeruginosa* (Sabath *et al.* 1965) and the Enterobacter strains reported here.

Penicillin is itself hydrolysed (albeit slowly) by these enzymes and this might explain why such high concentrations are required for induction. The kinetics of induction with methicillin, which is not attacked, may shed light on this observation.

If inducible β-lactamase in Gram-negative bacteria is a rarity, then it raises interesting questions about the evolution of these enzymes. Clearly some control of protein synthesis is necessary for the cell's economy and if this is not effected by repression, then what is the mechanism?

If non-inducible β-lactamase is the rule in Gram-negative bacilli, then either regulatory genes have undergone mutation, or they were never present. Although mutation in a regulatory locus should be a rare event, selection pressures encountered in hospitals could encourage survival of such mutants; this might explain the origin of the constitutive strain P99 or the non-inducible strains of *Escherichia coli* producing large amounts of enzyme described by Hamilton-Miller *et al.* (1965). Where low levels of β-lactamase were produced even in the presence of potential inducers, as in klebsiellas (unpublished observations), and in *E. coli* (Smith & Hamilton-Miller, 1963; Ayliffe, 1965), some form of regulation, presumably repression, is likely and induction cannot be ruled out just because the conditions required have not been determined.

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Use of the Coulter Counter to Measure Osmotic Effects on the Swelling of Mould Spores During Germination

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SUMMARY

The increase in size of *Trichoderma* (strain IMI 110150) spores during germination has been measured using the Coulter Counter. For sizing with this instrument, the spores were transferred from nutrient medium into dilute electrolyte solution and this resulted in an apparent delay in the onset of swelling. The length of this lag period was related to the concentration of saline electrolyte and not to the rate of swelling or the size of the spores.

INTRODUCTION

The swelling of mould spores, a recognized part of the germination process, has been the subject of several investigations reviewed by Sussman & Halvorsen (1966). The most recent contributions include those of Marchant & White (1966), Ekundayo, (1966), Barnes & Parker (1966*a*) and the contributors to the *Colston Papers* (1966).

The microscopical measurement of individual spores, sampled from the germination medium, has proved to be the method of choice for following this period of growth. Other techniques involve measurement of changes in dry weight (Marchant & White, 1966; Bartnicki-Garcia & Nickerson, 1962), respiration rates (Mandels, Levinson & Hyatt, 1956), packed cell volume (Mandels & Darby, 1953; Yanagita, 1957) and extinction of suspensions (Terui & Mochizuki, 1955).

The Coulter principle of size analysis (Coulter, 1956) involves the passage of a particle, suspended in an electrolyte solution, through a small orifice. At the same time an electric current is passed through the orifice and by displacing its own volume of electrolyte, the transient particle momentarily changes the resistance between the immersed electrodes. A small voltage pulse is produced, which is proportional in magnitude to the volume of the particle. The series of pulses, which are produced by the particles, suspended in a known volume of electrolyte, which pass through the orifice in a given time, are electronically amplified, scaled and counted.

The various models of the Coulter Counter and the techniques involved in their use have been described elsewhere (Barnes, Parker & Bradley, 1966). The instrument measures individual particles and offers advantages over optical methods in the greatly increased number of particles measured and in the minimization of the errors and tedium involved in size analysis.

The use of automatic methods of size analysis for measuring the swelling of mould spores during germination was suggested by Barnes (1964). In subsequent investigation the Coulter Counter proved the instrument of choice. The use of this instrument

to measure changes in volume or number of fungal cells has also been suggested by Mandels (1965).

The swelling of both mould and bacterial spores during germination is suppressed by antimicrobial agents (Mandels & Darby, 1953; Hitchins, Gould & Hurst, 1963). We have used the Coulter Counter to evaluate pharmaceutical preservatives by their effect upon spore swelling (Parker, Barnes & Bradley, 1966).

This paper reports on the use of the Coulter Counter for measuring the swelling of mould spores and on the osmotic phenomena observed.

METHODS

Coulter Counter models A and B were used with 50 μ orifices. Calibrations involved the use of two sources of monosized particles, polystyrene latex at 2·956 μ diameter (Dow Chemical Co.) and mould spores at 2·90 μ diameter (Coulter Electronics Ltd., Barnes & Parker, 1966*b*). The electrolyte consisted of solutions of sodium chloride A/R in glass distilled water and was cleaned by filtration through 0·45 Millipore filters.

Spore suspensions of *Trichoderma* sp. strain IMI 110150 from 21-day plate cultures were freshly prepared for each experiment and the spores were germinated in aerated 0·2% Malt Extract BP as described previously (Barnes & Parker, 1966*a*). An exception to this procedure was when spores which had previously been stored in aqueous suspension at 4° were used in investigating the effects of Chlorbutol (see Fig. 2). At intervals samples of the spores suspended in the nutrient medium were removed and 50 spores measured optically at magnification × 1000 using a microscope with projection head. At hourly intervals a sample of the same germinating spores was removed and diluted approximately 1/100 with the filtered electrolyte and the spores size-analyzed with the Coulter Counter. Thus the swelling of the same bulk sample of spores was determined simultaneously by the two methods, the spores being suspended in the nutrient medium for optical measurement and in saline solution for the Coulter analysis.

The experiments were repeated, alternating the Coulter and microscopical sizings. Duplicate experiments are shown in Fig. 1. In Fig. 1A the open and closed symbols represent duplicate results for 21-day spores in 1·75% NaCl. Figure 1B represents results from 21-day spores (closed symbols) and 28-day spores (open symbols) in 1·5% NaCl. The rate of swelling of the older spores was slower, a phenomenon which has previously been described (Barnes, 1964).

Chlorbutol, (2,2,2-trichloro-1,1-dimethyl-ethanol: Evans Medical Supplies Ltd., Liverpool), a preservative widely used in pharmaceutical preparations, was used to illustrate the performance of the Coulter Counter in assessing antifungal activity. Ten ml. each of double strength nutrient medium and preservative solutions of twice the required concentrations were mixed, and inoculated with 0·1 ml. of aqueous spore suspension which had been stored at 4°. Distilled water replaced the preservative solution in the control experiment. At intervals shown in Fig. 2 samples of the spores from the control and from each of the preservative systems were diluted with 0·9% NaCl electrolyte and sized on a model B Coulter Counter.

RESULTS

The Coulter Counter Model A results are in the form of a cumulative size distribution of the large number of spores counted and sized. A convenient measurement of spore size changes during germination is the change in the size corresponding to the half-count, i.e. the numerical average size. The model B instrument automatically plots a histogram of the spore size distribution, and the change in size value correspond-

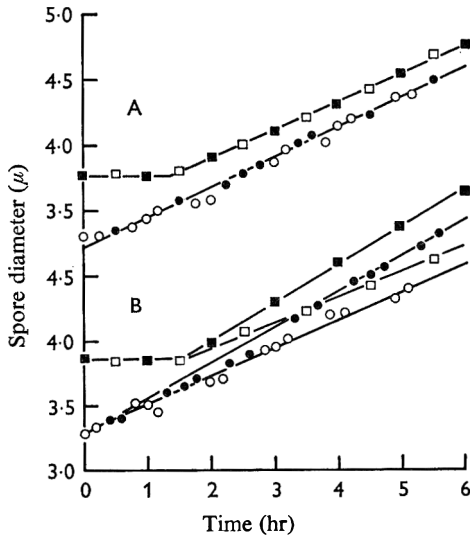

Fig. 1. Spore swelling measured microscopically and by the Coulter Counter. ■-□ Coulter Counter results. Size units are equivalent sphere diameters corresponding to the half count. ●-○ Optical measurements. Size units are average values of 50 spores. Open and closed symbols, duplicate experiments. Coulter Counter electrolytes: A, 1·75% (w/v) NaCl. B, 1·5% (w/v) NaCl.

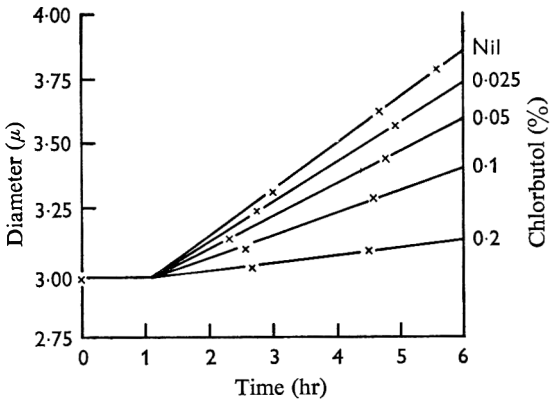

Fig. 2

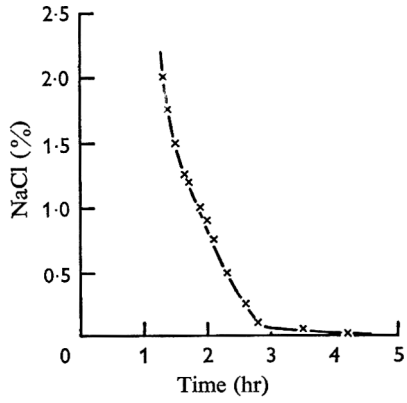

Fig. 3

Fig. 2. The effect of chlorbutol on swelling as measured by the Coulter Counter.
Fig. 3. Effect of NaCl concentration on time of onset of apparent swelling as measured by the Coulter Counter.

ing to the peak of the distribution has previously been used to follow spore swelling (Parker *et al.* 1966) and is used in Fig. 2.

Since the size distribution of *Trichoderma* sp. spores is skewed (Barnes & Parker, 1967) the peak size and the half-count size are not identical, and they differ from the arithmetic average size obtained from microscopical measurement. Comparison of the results obtained microscopically and by the Model A are shown in Fig. 1. The effect of using the different criteria of spore size is shown in the separate but parallel curves.

When spores sampled in Malt Extract were measured optically there was a linear increase with time in average diameter from the beginning of the experiment (Fig. 1 A). The same spores, suspended in 1·75% (w/v) NaCl and sized on the Coulter Counter showed a parallel swelling, but this did not commence until 1·37 hr. Figure 1 B shows that when 1·5% (w/v) NaCl was used as the electrolyte this value was 1·5 hr.

These experiments were repeated using a range of saline concentrations which was applicable to the instrument, i.e. from 2·0% (w/v) to 0·025% (w/v) NaCl. The times at which the spore swelling could be first detected were obtained and Fig. 3 shows these values plotted against the electrolyte concentration.

In order to obtain a size distribution with the Coulter Counter, about 5×10^5 spores were measured (cf. 50 spores per sample for optical measurements). The effect of measuring a far greater number of spores with the Coulter Counter is shown (Fig. 1) by the greatly reduced scatter of the experimental determinations. Results from this instrument allowed a rate of swelling to be determined which was not ambiguous, whereas the scatter of results associated with microscopical data must be interpreted by 'goodness-of-fit' techniques. Only two or three size analyses with the Coulter Counter are necessary to determine the rate of spore swelling illustrated in Fig. 2.

DISCUSSION

Ekundayo & Carlile (1964) found that a high osmotic pressure produced by the addition of approximately 19% (w/v) of sucrose in the germination medium did not affect the rate of swelling of the spores of *Rhizopus arrhizus*. The transfer of the spores of *Trichoderma* sp. into electrolyte solution for Coulter Counter sizing is essentially a transfer from the nutrient medium into a medium exhibiting lower osmotic pressure. This had no effect on the rate of swelling but altered the time at which swelling is first detected by the Coulter Counter.

Since the swelling of the same bulk sample of spores was measured simultaneously by the two methods, the two curves, for example, in Fig. 1 A, may be superimposed. This shows that the apparent delay in the onset of swelling when the spores were transferred to electrolyte solution, was caused by an immediate increase in the size of the spores due to the uptake of water. Germination swelling was only detected with the Coulter Counter after a period of swelling in the Malt Extract when, presumably, the osmotic pressure of the spore contents had reached a value isotonic with the concentration of saline electrolyte used. For this reason Fig. 3 may be a plot of the fall in isotonicity of the germinating spores with sodium chloride solutions.

Different rates of spore swelling were involved in the duplicate experiments comprising Fig. 1 B, but the change in size of the spores due to immersion in electrolyte solution, and also the time at which spore swelling was detected by the Coulter Counter, are constant. This is also illustrated in Fig. 2 where the rate of swelling varies in the presence of different concentrations of chlorbutol.

This phenomenon is explainable if the fall in isotonicity of the germinating spores is related only to the time of germination and not to the size of the spores or the rate of increase in size. It is remarkable that this relationship should hold, even when swelling is almost prevented by antifungal action and explanation is difficult.

These observations are in agreement with the work we have previously published (Barnes & Parker, 1966*a*), which showed that swelling during approximately the first hour of germination of freshly suspended *Trichoderma* sp. spores in malt extract was due to inanimate uptake of water. If spores are used which have previously been stored in water, this portion of the spore swelling should have already taken place. When the swelling of such spores was measured in 0·9% (w/v) NaCl electrolyte, the two hours delay (Fig. 3) before the apparent onset of swelling should be reduced. Figure 2 shows that this was reduced by 56 min.

The type of swelling curve found in this work has not been reported previously, and the osmotic phenomenon was not apparent when the swelling of *Bacillus subtilis* spores was measured using a similar technique (Parker *et al.* 1966; Parker & Barnes, 1967). The swelling of other mould spores as measured by the Coulter Counter is being investigated since these instruments are useful for the accurate measurement of the increase in size of spores during germination.

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The Isolation and Some Properties of Radiation-sensitive Mutants of *Micrococcus radiodurans*

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SUMMARY

Treatment of the radiation-resistant bacterium *Micrococcus radiodurans* with ultraviolet (u.v.) radiation and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine resulted in the isolation of two mutants highly sensitive to u.v. radiation. They were also sensitive to ionizing radiation and to the action of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. The concentrations of sulphydryl groups in bacteria of the wild type and the mutants were not significantly different. Although the mutants were more sensitive to mitomycin C than the wild type the resistance of the latter was low. It is suggested that the DNA repair mechanism in the wild type operates very efficiently for the removal of single strand damage but not for that which involves cross-linking.

INTRODUCTION

Micrococcus radiodurans, a pigmented non-sporing bacterium, is characterized by its extreme resistance to ionizing radiation (Anderson, Nordan, Cain, Parrish & Duggan, 1956) and to ultraviolet (u.v.) radiation (Duggan, Anderson, Elliker & Cain, 1959). The bacterial DNA, considered to be the radiation-sensitive target in other, more sensitive, bacteria, is not unusual in *M. radiodurans* either in its base composition or its quantity per cell (Moseley & Schein, 1964) and it is not exceptionally resistant to u.v. radiation damage as measured by the amount of the lethal photoproduct, thymine-dimer, formed on irradiation (Setlow & Duggan, 1964). There is now evidence for the existence in *M. radiodurans* of an efficient enzymic mechanism for the repair of u.v. damage, which excises thymine-dimers from its DNA with such efficiency that the eventual death of the bacteria appears to be due to other causes such as damage to deoxycytidine and protein (Setlow & Boling, 1965; Boling & Setlow, 1966). The repair mechanism is of the dark-repair type, similar to that found in u.v.-resistant strains of *Escherichia coli* (Setlow & Carrier, 1964; Boyce & Howard-Flanders, 1964*a*) but apparently operating with a much greater efficiency, since *M. radiodurans* can survive much higher doses of u.v. radiation. For example, the dose of u.v. radiation required to inactivate 90% of the organisms in a culture of the u.v.-resistant strain of *E. coli* K 12 AB 1157 is about 1000 ergs/mm^2 (Boyce & Howard-Flanders, 1964*a*) while 15,000 ergs/mm^2 is needed to achieve the same effect with *M. radiodurans*. There is also evidence that the resistance of *M. radiodurans* to ionizing radiation, which unlike u.v. radiation does not form thymine-dimers in DNA, is due to an enzymic repair mechanism for DNA damage (Moseley & Laser, 1965*a*; Dean, Feldschreiber & Lett, 1966). This mechanism

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appears to be essentially the same as that which operates for the repair of u.v. damage (Moseley & Laser, 1965*b*). The comparison of events which occur in u.v.-sensitive and u.v.-resistant strains of *E. coli* after u.v. radiation has greatly contributed to an understanding of the repair processes occurring in the resistant strains. Attempts have therefore been made to isolate radiation-sensitive mutants of *M. radiodurans* in order to establish by comparison the mechanism which confers on the wild type the unique property of being the vegetative bacterium which is most resistant to radiation.

METHODS

Organisms. The strain of *Micrococcus radiodurans* used was originally isolated by Anderson *et al.* (1956) and has been propagated in this laboratory for about 9 years. The bacteria grow in tetrads and contain a carotenoid pigment which gives colonies a salmon-pink appearance. The pigment can be extracted from dried bacteria with warm methanol and has an absorption peak at 475 mμ.

Media. TGYA broth for growth contained Bacto-Tryptone (Difco), 5 g.; glucose, 1 g.; yeast extract (Difco), 3 g.; aspartic acid, 2 g.; distilled water, 1 l., adjusted to pH 7·2 with NaOH. TGYA agar for colony counts and replica plating was made by solidifying this medium with 15 g. Bacto agar/litre.

A buffer solution was used for suspending organisms during irradiation. It contained: KH_2PO_4, 13·6 g.; Na_2SO_4, 2 g.; $MgSO_4.7H_2O$, 0·2 g.; $Ca(NO_3)_2.4H_2O$, 0·01 g.; $FeSO_4.7H_2O$, 0·5 mg.; distilled water to 1 l., adjusted to pH 7·2 with KOH. The same buffer was used for washing the bacteria and for dilution of suspensions for colony counts.

Preparation of organisms for irradiation. Colonies from an agar plate were inoculated into 12 ml. TGYA broth in L-tubes and the tubes shaken at 30° for 18 hr. The organisms were centrifuged down, washed and resuspended in buffer solution at a concentration of about 10^8 colony forming units/ml. Care was taken to break up clumps of organisms by homogenising the suspensions immediately before irradiation by using an MSE homogeniser (Cat. No. 7700).

U.v. irradiation was done by using a Hanovia germicidal lamp (Model 12). 5 ml. samples of washed bacterial suspension were irradiated in Petri dishes (9 cm. diam.) at a distance of 40 cm. from the lamp, the dose rate being 22·5 ergs/mm.^{2}/sec. The suspension was agitated during irradiation by means of a magnetic stirrer to prevent sedimentation of the bacteria and to maintain uniformity of the absorbed dose.

γ-irradiation was carried out in a ^{60}Co source at a dose rate of 16·7 Krad/min. Three ml. volumes of bacterial suspension were irradiated, oxygen being bubbled during the irradiation.

Isolation of mutants sensitive to u.v. radiation. Optimum conditions for the isolation of bacterial mutants usually result in the diminution of the colony count to about 0·1–1·0% of the initial count by treatment with the mutagen. However, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NG) has been found to give high mutation rates in *Escherichia coli* at concentrations which caused only a 50% loss of viability (Adelberg, Mandel & Chen, 1965). In contrast, very much higher concentrations of NG (500 μg./ml.) did not produce any loss of viability in *Micrococcus radiodurans* even after incubation for 3 hr. In view of the additive effect of ionising and u.v. damage (Moseley & Laser, 1965*b*) it was decided to combine u.v. radiation with application of the mutagen. This was done

by irradiating the bacteria with a non-lethal dose of u.v. radiation before exposing them to the mutagen. This combined treatment produced the desired decrease in viability with optimal chances of producing sensitive mutants.

Four ml. of a suspension (about 10^8 colony forming units/ml.) irradiated with 10,000 ergs/mm.^{2} of u.v. radiation were added to 5 ml. TGYA broth and the suspension shaken at 30° for 30 min. One ml. NG solution (5 mg./ml.) was added to a concentration of 500 μg./ml. After 45 min. further incubation the colony count was decreased to 0·1–1·0% of the original count. Samples (0·1 ml.) were diluted 100-fold in TGYA broth, to dilute out the mutagen, and shaken at 30° to allow one or two divisions to take place. Agar plates were then spread with dilutions of culture to give about 80–100 colonies/plate and incubated at 37° for 2 days. Two replicate copies of each plate were made by the felt-pad technique (Lederberg & Lederberg, 1952). One copy was irradiated under the u.v. lamp for 20 min. (a dose determined by sterilising small colonies of u.v.-sensitive *Salmonella typhimurium* CLT 22). After incubation for 24 hr. at 37° the non-irradiated and irradiated plates were compared for growth. Colonies on the non-irradiated plates which were absent from the irradiated plates were isolated and screened for u.v. sensitivity.

Resistance of Micrococcus radiodurans to the lethal action of NG and to mitomycin C. A sample (2·5 ml.) of an 18-hr culture of *M. radiodurans* was added to 7·5 ml. TGYA broth and shaken at 30° for 100 min. (about 1 generation time). NG or mitomycin C was added to 100 μg./ml. and 20 μg./ml., respectively, and 0·1 ml. samples removed at suitable time intervals, diluted in buffer, and colony counts made on TGYA agar plates.

Determination of sulphydryl-group concentration in Micrococcus radiodurans. The technique was based on that of Hamm & Hofmann (1965). To 1 ml. of a thick suspension of organisms (equiv. 50–70 mg. dry wt), 35 ml. of 8 M-urea and a slight excess of $AgNO_3$ (3·5 μ mole) were added. After stirring at room temperature for 1 hr to allow complete reaction between the SH-groups and $AgNO_3$, 4·5 μ mole of glutathione was added to give a slight excess of SH-groups. The excess of glutathione (as SH) was then titrated amperometrically with 10^{-3} M-$AgNO_3$.

RESULTS

Isolation of mutants

Two mutants of wild-type *Micrococcus radiodurans* which were sensitive to u.v. radiation were isolated. The two mutants are referred to as *M. radiodurans* UV 17 and *M. radiodurans* UV 38. It was necessary before studying the new strains to show that they were mutants of *M. radiodurans* and not contaminants. The colonies of all three strains, wild type, UV 17 and UV 38 were similar in size and appearance, and all contained the carotenoid pigment which shows an absorption peak at 475 mμ. The concentration of pigment/unit dry wt organism was not significantly different in the three strains. Morphologically the organisms were identical and had the same mode of division which led to the formation of tetrads. Comparison of turbidity measurements with colony counts over the growth cycle gave similar plots, indicating that the dimensions of the organisms were similar.

Growth rates in TGYA broth were measured by using turbidity and colony count methods. In log-phase growth at 30° with adequate aeration the generation time for

the wild type was 90 min.; UV 17 had the same generation time, but that of UV 38 was 150 min., i.e. about 1·7 times longer. During the study of mutant UV 17 another mutant arose spontaneously which was non-pigmented but which had all the other properties of UV 17, e.g. identical cell morphology, growth rate, and radiation sensitivity. This strain has been called *M. radiodurans* UV 17W.

Radiation resistance

The dose response curves of the two mutants to u.v. radiation, compared with that of the wild type, are plotted in Fig. 1 which shows an enormous increase in sensitivity. The dose response curves to γ-radiation (Fig. 2) shows that even though the mutants were isolated on the basis of their u.v. sensitivity they were also sensitive to ionizing

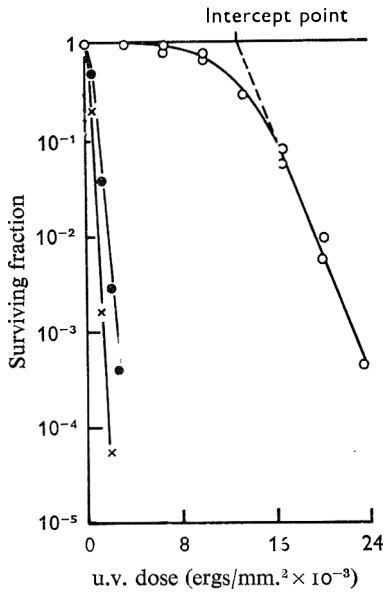

Fig. 1

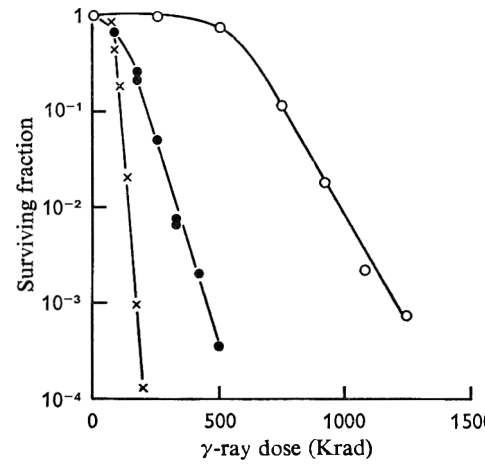

Fig. 2

Fig. 1. Ultraviolet (u.v.) irradiation survival curves of *Micrococcus radiodurans* (○) and the mutants UV 17 (×) and UV 38 (●). The exponential portion of the survival curve of *M. radiodurans* has been extrapolated to unit survival to indicate the derivation of the intercept value used in Table 1.

Fig. 2. γ-irradiation survival curves of *M. radiodurans* (○), UV 17 (×) and UV 38 (●).

radiation. All the survival curves were sigmoidal. The shapes of the dose response curves have been defined by their intercept number, which is the dose obtained by extrapolating the exponential part of the curve to unit survival (see Fig. 1). A summary of the results is given in Table 1.

Although the mutants were much more sensitive than the wild type, the increase in sensitivity to both types of radiation was not the same. A direct comparison of the survival curves is complicated by the fact that, with one exception, the increase in the exponential slope was not proportional to the reduction in the shoulder. Thus the reduction in the length of shoulder of strain UV 38 was much greater (28-fold for u.v. and five-fold for γ-radiation) than the increase in exponential slope, viz. five-fold for u.v.-radiation and two-fold for γ-radiation. Nevertheless, the data show that for

Table 1. *Analysis of the dose-response curves shown in Figs. 1 and 2*

The D 10 value is the dose required to decrease the colony count of a culture by 90% on the exponential part of the survival curve. The intercept value, in terms of dose, is obtained by extrapolating the exponential part of the curve to unit survival. The ratios are derived from the D 10 and intercept values of the various strains divided by those of the wild type.

	u.v. radiation dose response curves				γ-radiation dose response curves			
Strain	D 10 (ergs/mm.²)	Ratio	Intercept (ergs/mm.²)	Ratio	D 10 (Kr)	Ratio	Intercept (Kr)	Ratio
Wild type	3,400	1·0	12,800	1·0	210	1·0	525	1·0
UV 17	340	0·1	450	0·035	30	0·14	75	0·14
UV 38	680	0·2	450	0·035	105	0·5	100	0·2

strain UV 38 the increase in sensitivity to u.v. radiation was greater than that for γ-radiation. The same is true for strain UV 17, the reduction in the shoulder being 28-fold for u.v. radiation and 7-fold for γ-radiation, while the exponential slope increased by factors of 10 and 7 respectively.

The lethal effect of N-methyl-N'-nitro-N-nitrosoguanidine (NG)

Wild type *Micrococcus radiodurans* was completely resistant to the effect of 100 μg. NG/ml., showing no loss of viability after 80 min. of incubation. The radiation-sensitive mutants UV 17 and UV 38 were sensitive to the lethal action of NG, less than 10^{-3} of their populations surviving after 10 and 15 min. of incubation, respectively (Fig. 3). From this it follows that the increase in sensitivity of the mutants to NG was greater than that towards radiation. It was noted that practically all the colonies derived from wild-type organisms which had been incubated in the presence of NG 100 μg./ml. for 80 min. showed abnormalities, e.g. sectoring caused by partial loss of pigment, roughness, growth-rate variations, leading to the formation of very irregular edges to the colonies. On the other hand, colonies derived from surviving organisms of strains UV 17 and UV 38 were normal as compared with colonies from untreated organisms.

The lethal effect of mitomycin C

Figure 4 shows the lethal effect of mitomycin C at a concentration of 20 μg./ml. on *Micrococcus radiodurans* and its mutants UV 17 and UV 38. The radiation-sensitive mutants were more sensitive to the action of mitomycin C than the wild type but the disparity was less than that for the lethal effect of u.v. radiation.

Sulphydryl-group content

Three samples of each bacterial strain were analysed for sulphydryl group content. Values obtained were 21, 24, and 27 μmole SH/g. dry wt of wild type, strain UV 17 and strain UV 38, respectively. The dry weight of a colony-forming unit of *Micrococcus radiodurans* obtained previously was $2{\cdot}5 \times 10^{-12}$ g. (Moseley & Laser, 1965*a*) which gives values of 0·5, 0·6, and $0{\cdot}7 \times 10^{-16}$ mole SH/colony-forming unit for the three strains.

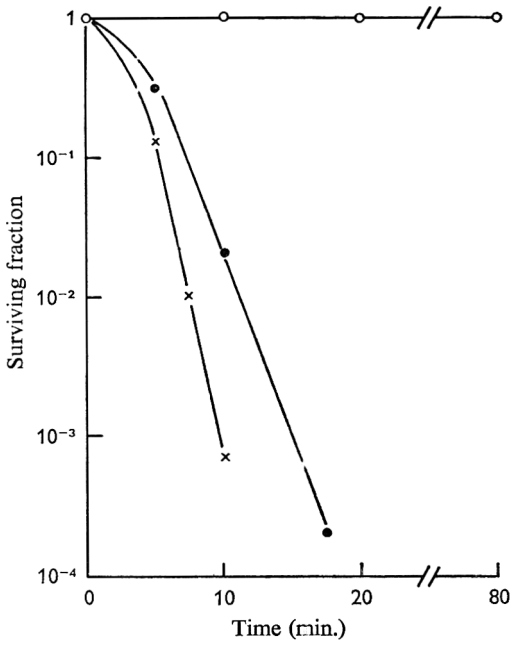

Fig. 3

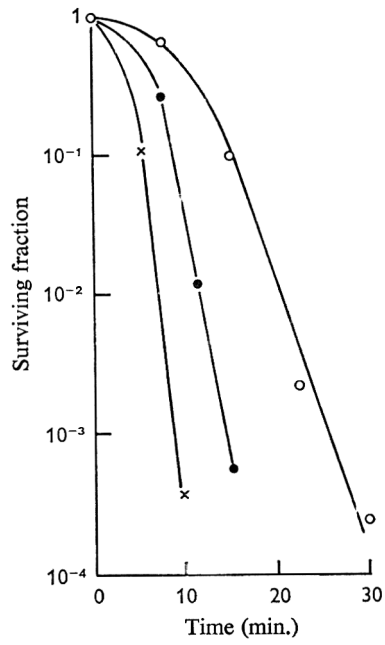

Fig. 4

Fig. 3. Resistance of *M. radiodurans* (○), UV 17 (×) and UV 38 (●) to incubation in NG 100 μg./ml.

Fig. 4. Resistance of *M. radiodurans* (○), UV 17 (×) and UV 38 (●) to incubation in mitomycin C 20 μg./ml.

DISCUSSION

The fact that *Micrococcus radiodurans* is the most resistant vegetative bacterium, so far investigated, to u.v. radiation as well as ionizing radiation has often been overlooked, and explanations have been sought for its resistance to ionizing radiation which have no relevance to its resistance to u.v. radiation. Some explanations have postulated that an intracellular carotenoid pigment (Kilburn, Bellamy & Terni, 1958) or a sulphydryl compound (Bruce, 1964; Bruce & Malchman, 1965) act as protectors. The isolation of non-pigmented mutants of *M. radiodurans* which have the same resistance as the wild type of ionizing radiation indicated that the pigment is not responsible for such resistance (Moseley & Laser, 1965*a*) but it could not be excluded that pigment precursors were present which acted as energy-transfer substances. This possibility has now been excluded by the isolation of pigmented sensitive mutants.

The value of $0{\cdot}5$–$0{\cdot}6 \times 10^{-15}$ mole SH/colony-forming unit is in reasonable agreement with that obtained by Bruce & Malchman (1965) of $0{\cdot}8$–$2{\cdot}0 \times 10^{-16}$ mole/organism based on the binding of *p*-hydroxymercuribenzoate. However, this concentration of sulphydryl groups is present in the radiation-sensitive organisms as well as in the radiation-resistant organisms. The mere presence of sulphydryl groups is not an indication of the presence of an intracellular protective compound able to confer such high resistance as is shown by the wild-type *Micrococcus radiodurans*. However, some of the residual resistance to ionizing radiation in the sensitive strains may yet be due to the presence of sulphydryl groups and thus account for the fact that they

are slightly less sensitive to ionizing radiation than to u.v. radiaton. This is a very small part of the total resistance of the wild-type *M. radiodurans* to ionizing radiation, the major part of which is due to the presence of a repair mechanism.

The dark repair system which operates in u.v. resistant strains of *Escherichia coli* does not only recognize damage to DNA of the thymine-dimer type. The excision of defective bases and the incorporation of new ones follows treatment of organisms with the bifunctional alkylating agent nitrogen mustard (Hanawalt & Haynes, 1965) and with NG (Hanawalt, 1966). Thus the precise base defect appears to be less important than some associated secondary structural alteration in the phosphodiester backbone of the DNA. This situation is also true for *Micrococcus radiodurans*. Its remarkable capacity to survive extremely high doses of ionizing radiation and of u.v. radiation is complemented by its resistance to the decay of radioactive phosphorus (^{32}P) incorporated in its DNA and to the action of NG, which probably acts as a monofunctional alkylating agent. *Micrococcus radiodurans* organisms remain viable even after 50,000 ^{32}P disintegrations/nucleus, as compared with values of 10–50 for *E. coli* (M. Swann, personal communication). Since *M. radiodurans* has such great resistance to radiation and to compounds which cause DNA defects, it is surprising that it is sensitive to mitomycin C which cross-links DNA *in vivo* (Iyer & Szybalski, 1963) and which functions partially as a bifunctional alkylating agent (Iyer & Szybalski, 1964). Although wild-type *M. radiodurans* is more resistant to mitomycin C than are the radiation-sensitive mutants, the order of resistance is quite low, being the same as that shown by u.v.-resistant strains of *E. coli* (Boyce & Howard-Flanders, 1964*b*). This suggests that *M. radiodurans* has a repair system of extremely high efficiency for damage within one or both strands of the DNA, but which does not operate for damage involving cross-linkage. The higher resistance of wild-type *M. radiodurans* as compared with that of the sensitive mutants need not be interpreted in terms of removal of cross-links since only 1 out of 5 to 10 mitomycin C molecules participates in the formation of cross-links while the others react with one DNA strand only (Iyer & Szybalski, 1964; Weissbach & Lisio, 1965). This suggests that the differential sensitivity of the three strains of *M. radiodurans* to mitomycin C reflects the inability of the radiation-sensitive strains to repair monofunctional alkylating damage which the wild type repairs very efficiently (hence its high resistance to NG) but is inactivated by cross-link damage. Preliminary biochemical studies indicate that the radiation-sensitive mutants and wild-type *M. radiodurans* are able to excise u.v. radiation-induced thymine dimers from their DNA and are able to incorporate fresh bases. Their sensitivity appears to be due to the inability to control the excision process (Moseley, 1967).

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The Metabolism of Acetate by the Blue-green Algae, *Anabaena variabilis* and *Anacystis nidulans*

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SUMMARY

The utilization of acetate by blue-green algae was examined and the activities of enzymes involved in its metabolism measured. Although acetate did not stimulate the endogenous respiration of these organisms, the oxidation of acetate was followed by the rate of release of [^{14}C] carbon dioxide from [1-^{14}C] and [2-^{14}C] sodium acetate. Similarly, sodium acetate did not alter the rate of growth of *Anabaena variabilis* and *Anacystis nidulans* but in *A. variabilis* it was found to contribute 7·2 % of the dry weight when cultures were gassed with air + CO_2 (95 + 5, v/v), and 16·9 % when gassed with air alone. The presence of acetate in the growth medium did not alter the activity of the acetate-activating enzymes, glyoxylate cycle enzymes or two tricarboxylic acid cycle enzymes. The failure to show enzyme adaption by these organisms when supplied with an exogeneous substrate is discussed in relation to their hitherto apparently autotrophic nature.

INTRODUCTION

The blue-green algae, in spite of diverse ecological distribution, have markedly uniform nutrition. Most of the species examined are considered to be strict photoautotrophs (Allen, 1952) and their failure to respond to external carbon sources other than carbon dioxide has been noted by many workers. Exceptions to this statement include *Tolypothrix tenius*, the growth of which is stimulated by glucose (Kiyohara *et al.* 1960; 1962) and *Chlorogloea fritschii*, which has been reported to grow in the dark on sucrose albeit the growth rate was expressed in terms of months rather than days (Fay, 1965). The incorporation of [^{14}C] acetate into *Nostoc muscorum* and transformation into lipids has been known for some years (Allison *et al.* 1953); Hoare & Moore (1965) described the photoassimilation of acetate by three blue-green algae and also showed major incorporation to be into the lipid fraction. The fact that *Chlorogloea fritschii* forms poly-β-hydroxybutyrate only when grown in the presence of acetate, also indicates the utilization of this substrate by a blue-green alga (Carr, 1966). We therefore decided to examine in the blue-green algae the metabolism of acetate at the whole organism and enzymic levels. Some preliminary results of this investigation have already been reported (Carr & Pearce, 1966; Pearce & Carr, 1966).

METHODS

Organisms. We are grateful to the following for gifts of the cultures of the blue-green algae used: *Anabaena variabilis* (Kützing) from Professor J. Myers, Department of Zoology, University of Texas, Austin, Texas; *Anacystis nidulans* from the collection

of Dr M. B. Allen through the courtesy of Dr A. A. Horton, Department of Biochemistry, University of Birmingham; *Chlorogloea fritschii* from Professor G. E. Fogg, Department of Botany, Westfield College, University of London, London. All organisms were maintained on agar (2%) slopes of the mineral salt medium (see below) supplemented with sodium acetate (10 mM) and yeast extract (Difco, 1%).

Growth. The algae were grown on a mineral salt medium (Medium C, Kratz & Myers, 1955*a*) to which $NaHCO_3$ (0·05%) had been added. Sodium acetate (20 mM) was added where indicated in the text. Cultures for experimental purposes were grown in Carrel flasks (penicillin pots) at 34° in 500 ml. medium illuminated by 30 W warm white daylight strip lights and gassed with air+CO_2 (95+5, by vol.) as previously described (Carr & Hallaway, 1965). The growth of each organism was determined turbidimetrically in an EEL colorimeter by comparison with a previously prepared calibration curve relating EEL reading to dry weight of organism.

Incorporation of [^{14}C] *labelled acetate.* Experiments involving [U-^{14}C]acetate were carried out in 20 ml. volumes but otherwise under the conditions described above. [^{14}C]carbon dioxide was released from [^{14}C]sodium bicarbonate by 5 N-HCl and pumped through the algal cultures in a closed system which recycled the gas phase. The extent of isotopic incorporation was measured in a harvested and thoroughly washed (six times) suspension by counting, after drying, at infinite thinness in a Nuclear-Chicago Gas-Flow Automatic Planchette Counter. Each sample was counted in duplicate to an accuracy of 2%.

Manometry. Logarithmic phase cultures were harvested, the organism, washed and resuspended in sterile growth medium (pH 7·4) or 0·1 M-potassium phosphate buffer (pH 7·0) and oxygen uptake measured at 34° by the direct procedure and expressed as Q_{O_2} (μl./mg. dry wt/hr) with and without the addition of sodium acetate (30 mM). In experiments involving [1-^{14}C]acetate and [2-^{14}C]acetate double side-arm Warburg flasks were used which contained hyamine hydroxide (Snyder & Godfrey, 1961) in the centre well instead of NaOH, sodium acetate [^{14}C] in one side arm and 0·5 ml. sodium dodecylsulphate (10%) in the other. At various times the respiration was stopped by adding sodium dodecylsulphate. After 45 min., to allow complete absorption of carbon dioxide by the hyamine hydroxide, the contents of the centre well were transferred by syringe to 5 ml. of scintillation fluid and counted in a Packard Tri-Carb Liquid Scintillation Spectrometer Series 314E to an accuracy of at least 2%.

Preparation of cell-free extracts. Organisms were harvested during the logarithmic phase of growth, washed once with distilled water and resuspended in 0·1 M-phosphate buffer (pH 7·0) to a final concentration equiv. 30–60 mg. dry wt/ml. Organisms were broken by (*a*) extrusion through a French pressure cell at 10,000 lb./sq. in., or (*b*) ultrasonic disintegration in an M.S.E. 60 W., 20 Kc/sec. sonic disintegrator for three 45-sec. periods. In either case a temperature of 0–2° was maintained and enzyme activities were similar in extracts prepared by either means. Cell-wall debris and unbroken organisms were removed by centrifugation at 10,000 *g* for 15 min. at 0°, yielding an intensely green supernatant fluid of cell-free extract.

Estimations

Acetyl-CoA synthetase. [EC. 6.2.1.1]. This was assayed by the procedure of Jones & Lipmann (1955). The reaction mixture contained, in μmoles, potassium phosphate buffer (pH 7·0), 50; $MgCl_2$, 10; cysteine, 20; coenzyme A, 0·05; ATP, 5; freshly

neutralized hydroxylamine, 1000; sodium acetate, 200; in a total volume 1·5 ml. The reaction was done at 34° and initiated by the adding of cell-free extract containing 5–10 mg. protein. Hydroxamic acid formation was estimated by the increase in extinction at 540 mμ after the adding of the $FeCl_3$ reagent.

Acetate kinase. [E.C. 2.7.2.1]. The synthesis of acetylphosphate was determined, after reaction with neutralized hydroxylamine, by the formation of a $FeCl_6$ complex as above. The composition of the reaction mixture was the same as that for the assay of acetyl-CoA synthetase except that coenzyme A was omitted.

Phosphotransacetylase [E.C. 2.3.1.8]. This enzyme was measured by following the disappearance of acetylphosphate when incubated with coenzyme A in the presence of arsenate (Stadtman, 1952). The reaction mixture contained, in μmoles, potassium phosphate buffer (pH 7·0), 50; dilithium acetylphosphate, 2; coenzyme A, 0·05; cysteine, 10; potassium arsenate, 50; 5–10 mg. protein in a total volume of 2·0 ml. At intervals the residual acetylphosphate was determined by the hydroxamic acid procedure of Lipmann & Tuttle (1945).

Citrate synthase [E.C. 4.1.3.7] Where acetate kinase and phosphotransacetylase were the means of activating acetate (i.e. in extracts of *Anabaena variabilis*) citrate synthase was measured by the disappearance of acetylphosphate in the presence of coenzyme A and oxaloacetate using the procedure of Ochoa (1955). Acetylphosphate was estimated as the hydroxamic acid and an appropriate control permitted a correction for the small amount of acetyl-CoA metabolized by deacylation. In extracts of *Anacystis nidulans* in which acetate is activated directly by acetyl-CoA synthetase a different procedure was adopted (Srere, Brazil & Gonen, 1963). This involved the assay of coenzyme A released from acetyl-CoA on condensation with oxaloacetate. The sulphydryl group of coenzyme A reacts with 5,5′-dithiobis-[2-nitrobenzoic acid] to yield 2-nitro-5-mercaptide benzoate which has an extinction maximum at 412 mμ. The reaction mixture contained, in μmoles, potassium phosphate buffer (pH 7·0), 200; acetyl-coenzyme A, 0·05; $MgCl_2$, 10; cysteine, 20; 5,5-′dithiobis-[2-nitrobenzoic acid], 20; 3–5 mg. protein in a total volume of 2·5 ml. The reaction was initiated by adding 10 μmoles oxaloacetate.

Isocitrate dehydrogenase [E.C. 1.1.1.42]. This was assayed by following the rate of reduction of NADP in the presence of isocitrate according to the method of Ochoa (1948). The reaction mixture contained, in μmoles, potassium phosphate buffer (pH 7·0), 200; $MnCl_2$, 5; NADP, 0·5; extract (containing 3–5 mg. protein) in a total volume of 2·5 ml. After the addition of 10 μmoles isocitrate the rate of reduction of NADP was followed at 340 mμ.

Isocitrate lyase [E.C. 4.1.3.1]. The incubation of isocitrate with algal extracts results in the formation of keto acid phenylhydrazones linearly with time. The identity of the keto acid formed was examined by incubation in the presence of semicarbazide and conversion of the resulting semicarbazone to the 2,4-dinitrophenylhydrazone, Extraction into ethyl acetate by the method of Friedemann & Haugen (1943) and chromatography showed the presence of the 2,4-dinitrophenylhydrazones of glyoxylate and α-ketoglutarate. The production of glyoxylate from isocitrate was confirmed by formation of a compound spectrally identical with that of 1,5-diphenylformazan carboxylic acid which is produced from glyoxylate phenylhydrazone under acid conditions (Kramer, Klein & Baselice, 1959). We concluded, therefore, that in our extracts the standard procedure of Dixon & Kornberg (1959) did not eliminate the

action of isocitrate dehydrogenase. Attempts to remove the endogenous pyridine nucleotide or to maintain it in a reduced form did not satisfactorily resolve the problem. Physical separation of isocitrate lyase and isocitrate dehydrogenase was achieved by centrifugation of the crude cell-free extract at 78,000 *g* for 20 hr. The fraction containing isocitrate lyase activity did not contain any isocitrate dehydrogenase activity. The isocitrate lyase activity of this fraction was assayed according to Dixon & Kornberg (1959). The reaction mixture contained, in μmoles, potassium phosphate buffer (pH 7·0), 200; cysteine, 20; $MgCl_2$, 10; phenylhydrazine, 20; and 0·3–0·5 mg. protein in a total volume of 2·5 ml. The reaction was initiated by the addition of 20 μmoles DL-isocitrate and the formation of glyoxylate phenylhydrazone followed at 324 mμ.

Malate synthase [E.C. 4.1.3.2]. With the Unicam S.P. 700 recording spectrophotometer and cell-free extracts of blue-green algae it was not possible to assay this enzyme by the standard procedure (Dixon & Kornberg, 1959) based on the decline of extinction at 232 mμ due to the disappearance of the thiolester bond of acetyl-CoA. Accordingly, a method was devised based on the rate of disappearance of glyoxylate (measured as its phenylhydrazone at 324 mμ) when incubated with acetyl-CoA or ATP, coenzyme A and sodium acetate. When the cell-free extract was treated with Dowex-1-×4 (50–100 mesh; Cl—form), which removed endogenous coenzyme A, there was no decrease in the amount of glyoxylate in the reaction mixture in the absence of added coenzyme A. A series of $5 \times \frac{5}{8}$ in. test tubes contained, in μmoles, potassium phosphate buffer (pH 7·0) 50; $MgCl_2$, 10; cysteine, 20; coenzyme A, 0·05; ATP, 5; sodium acetate, 200; sodium glyoxylate, 0·06; in a total volume of 2·0 ml. The assay was done at 34° and initiated by the addition of cell-free extract containing 3.7 mg. protein. The enzymic reaction was stopped at intervals (5, 10, 15, 20, 30, 40 min.) by adding 1 ml. 10% (w/v) trichloroacetic acid and 0·1 ml. phenylhydrazine HCl (0·2 M) and the extinction at 324 mμ determined after removal of the precipitated protein.

Protein estimation. The amount of protein in cell-free extracts was determined colorimetrically by a method based on the biuret reaction (Layne, 1957). The photosynthetic pigments were removed prior to protein estimation by hot acid-ethanol (Vernon & Kamen, 1953). The colour produced was determined at 550 mμ and compared with a standard of crystalline bovine serum albumen.

Chemicals. ATP, coenzyme A, NADP and NAD were obtained from C. F. Boehringer Ltd. (Mannheim, Germany); dilithium acetyl phosphate and 5,5′-dithiobis-[2-nitrobenzoic acid] from the Sigma London Chemical Company Ltd. (12 Lettice St., London, S.W.6); sodium dodecylsulphate from K & K Laboratories, Inc. (177–10 93rd Avenue, Jamaica 33, New York); hyamine hydroxide (molar solution in methanol) from Packard Instrument Ltd. (10–12 St. Johns Road, Wembley, Middlesex). All other chemicals were purchased from British Drug Houses Ltd. (Poole, Dorset) and were the purest commercial grade available. Radioactive sodium acetate ([1-^{14}C], [2-^{14}C] and [U-^{14}C]), and [2-^{14}C]sodium glyoxylate were purchased from The Radiochemical Centre, Amersham, Buckinghamshire.

RESULTS

Growth and respiratory studies

Short-term experiments. When the minimal salt (autotrophic) medium was supplemented with sodium acetate (20 mM) neither *Anabaena variabilis* nor *Anasystis nidulans* exhibited a significantly higher rate of growth. Variation of the concentration of sodium acetate (10–50 mM) was without effect and attempts to grow *A. variabilis* in the presence of sodium acetate and absence of carbon dioxide were unsuccessful. The failure of acetate and other substrates to stimulate respiration in blue-green algae has been known for some time (Kratz & Myers, 1955*b*) and in our experiments *A. variabilis* was no exception. Over a range of pH values (4·3–8·0) the endogenous rate of respiration of this organism was not significantly affected by the addition of sodium acetate to a concentration of 30 mM. The oxygen uptake by *A. nidulans* was slightly stimulated by the addition of glucose. Comparison of respiratory rates in the mineral salt medium and 0·1 M-potassium phosphate buffer (pH 7·0) showed that the growth medium was a superior environment for respiration by suspensions of washed organisms. When the organisms were starved of carbon dioxide for 4 hr immediately before harvesting, the rate of endogenous respiration was decreased; the rate of respiration was largely restored by the addition of sodium acetate or glucose; other organic compounds had less effect (Table 1).

Table 1. *Effect of various organic compounds on the respiration of Anabaena variabilis and Anacystis nidulans*

		Q_{O_2}	
Organism	Addition [30 mM]	*a**	*b**
A. variabilis	None	12·3	3·08
	Sodium acetate	11·7	2·9
	Glucose	13·1	3·5
	Sodium pyruvate	12·7	3·2
	Sucrose	11·2	3·17
A. variabilis (starved of CO_2 for 4 hr before harvest)	None	3·62	—
	Sodium acetate	11·8	—
	Glucose	8·63	—
	Sucrose	6·4	—
	Sodium pyruvate	4·1	—
A. nidulans	None	—	6·8
	Sodium acetate	—	8·9
	Glucose	—	9·8

* (*a*) manometry in minimal salt medium; (*b*) manometry in 0·1 M-potassium phsphate buffer (pH 7·0).

The metabolism of [1-^{14}C]acetate and [2-^{14}C]acetate was examined by measurement of the [^{14}C]carbon dioxide evolved during respiration (Fig. 1). The more rapid release of [^{14}C]carbon dioxide from [1-^{14}C]acetate is consistent with the operation of a tricarboyxlic acid cycle. However, the ratio of radioactivity from the carboxyl group as compared with the methyl group was somewhat greater than expected. This may indicate the presence of another route of acetate oxidation apart from the tricarboxylic acid cycle. The marked alteration in the rate of [^{14}C]carbon dioxide production from [1-^{14}C]acetate after about 1 hr may be due to possible direction of acetate into another

pathway or to the exhaustion of C_4 intermediates. The corollary of these results are shown in Fig. 2, which shows that [2-^{14}C]acetate was more effectively incorporated in cellular material of *A. variabilis* than was [1-^{14}C]acetate when incubated under identical conditions to those employed in the manometric experiments. It is noteworthy that the uptake of [^{14}C]acetate over the first hour was the same irrespective of light or darkness or the position of the labelled carbon in the acetate. This observation was consistent over three experiments and may be due to a period of equilibration where the rate of entry of the acetate molecules was the same in each case and was great enough to mask the subsequent metabolism of the acetate.

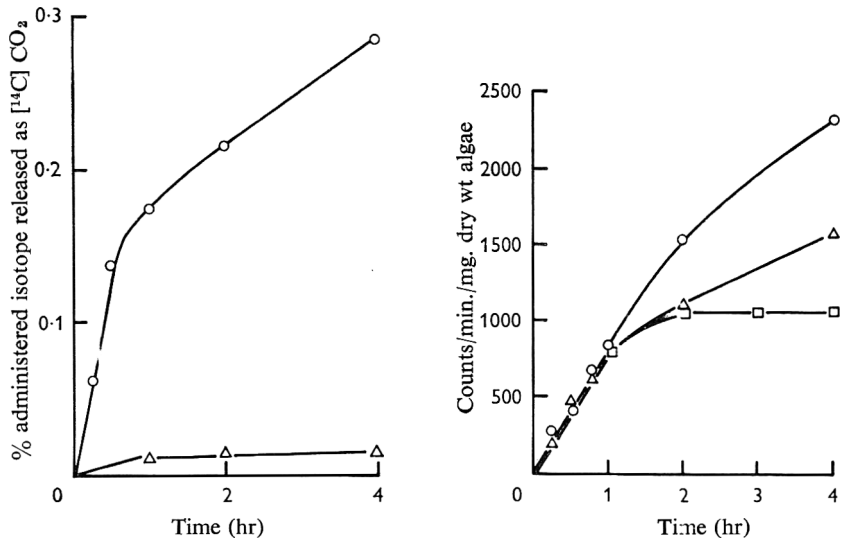

Fig. 1. The rate of release of [^{14}C] CO_2 from [1-^{14}C]acetate and [2-^{14}C]acetate by *Anabaena variabilis* in the dark. The experiment was done as described in Methods. To the suspensions of algae (equiv. 20 mg. dry wt/ml.) were added 100 μmoles sodium acetate containing 2μC [2-^{14}C]sodium acetate (—○—○—), or 2 μC [1-^{14}C]sodium acetate (—△—△—).

Fig. 2. Degree of incorporation of [1-^{14}C]acetate and [2-^{14}C]acetate into *Anabaena variabilis* in the light and the dark. The experiment was done in 150 ml. conical flasks containing 20 ml. volumes of medium C supplemented with sodium acetate. The flasks were incubated at 34°, gently shaken and illuminated by a 100 W tungsten lamp at a distance of 9 inches. The rates of incorporation of [2-^{14}C]acetate in the light (—○—○—), and in the dark (—□—□—), are compared with the rate of [1-^{14}C]acetate in the light (—△—△—).

Long-term experiments. Although there was no stimulation of the growth or respiration of *Anabaena variabilis* by the addition of sodium acetate, the entry of [U-^{14}C]acetate into the organisms was shown and its incorporation measured. After 7 days' growth, 7·2% of the total dry weight of *A. variabilis* was derived from the [U-^{14}C]acetate supplementing the growth medium. When the normal gas phase of air + CO_2 (95 + 5, v/v) was replaced by one of air alone, [U-^{14}C]acetate accounted for 16·7% of the total dry weight of organism. These figures indicate the degree of acetate assimilation and do not take into account any respired [U-^{14}C]acetate. Further evidence that exogenous sodium acetate was being appreciably incorporated into *A. variabilis* was provided by the depression of [^{14}C]-CO_2 incorporation into growing cultures when the growth medium was supplemented with 20 mM-sodium acetate.

Enzymic activities

In agreement with Hoare & Moore (1965) we found that *Anacystis nidulans* activated acetate directly by acetyl-CoA synthetase, this process being dependent on the presence of CoA. The same enzyme was found in *Chlorogloea fritschii*. *Anabaena variabilis*, however, formed acet-hydroxamate in the absence of coenzyme-A, and the formation of acetyl-CoA was mediated by the two enzymes, acetate kinase and phosphotransacetylase. The identity of the enzymic product was examined by co-chromatography of the hydroxamate formed with chemically prepared acet-hydroxamate. After growth in the presence of sodium acetate (20 mM), the specific activities of the enzymes were measured in cell-free extracts and compared with those obtained with cell-free extracts of autotrophically grown organisms (Table 2). There was no appreciable difference in any of the enzyme activities measured. The estimation of acetyl-CoA

Table 2. *Enzymes of acetate activation*

Enzyme activities are expressed in mμmoles/min./mg. protein and are the means of several determinations.

Organism	Growth in presence 20mM-acetate	Acetate kinase	Acetyl-CoA synthetase	Phospho-transacetylase
		mμmoles/min./mg. protein		
Anabaena variabilis	+	4·5	0	1·7
	−	4·1	0	1·4
Anacystis nidulans	+	0	3·0	n.t.
	−	0	3·1	n.t.
Chlorogloea fritschii	+	0	3·2*	n.t.
			1·1*	
	−	0	2·9*	n.t.
			3·0*	
			1·4*	

* one determination only; n.t., not tested.

synthetase in *C. fritschii* preparations gave variable results; a possible explanation of this was the tendency of the organism to clump during growth, thus preventing the development of a uniform culture. The effect of decreasing the amount of CO_2 available to *A. variabilis* was examined in the presence and absence of sodium acetate (20 mM). The growth rate was decreased when the normal gas mixture (air + CO_2, 95 + 5, v/v) was replaced by air alone. Growth under these conditions was not stimulated by sodium acetate, neither was acetate kinase activity increased.

The two key enzymes of the glyoxylate by-pass were measured as described in Methods. It was necessary to separate isocitrate lyase from the relatively highly active isocitrate dehydrogenease in extracts of *Anabaena variabilis* and *Anacystis nidulans*; this was achieved by centrifugation at 78,000 *g* for 20 hr. This resulted in the formation of two bands in the supernatant fluid of the extract; an upper yellow region which was shown to contain isocitrate lyase activity but no isocitrate dehydrogenase activity, and a larger phycocyanin-containing region which held most of the isocitrate dehydrogenase activity. The absence of NADP reduction in the presence of isocitrate by the yellow fraction is shown in Fig. 3*a*, indicating that this fraction did not possess isocitrate dehydrogenase activity. However, the presence of isocitrate lyase activity in

the yellow fraction was indicated by the formation of a phenylhydrazone, absorbing at 324 mμ, when isocitrate was added (Fig. 3*b*.) This enzyme was specific for NADP, no activity being shown with NAD. The isocitrate lyase activity was further characterized by the inhibition effected by sodium succinate (Fig. 4) and noted previously (Smith & Gunsalus, 1957). Malate synthase activity was assayed as described in Methods by following the rate of glyoxylate disappearance in the presence of an acetyl-CoA forming system (Fig. 5). When coenzyme A was omitted from the reaction mixture and endogenous coenzyme A removed from the extract by Dowex treatment there was no decline in the amount of glyoxylate. Anaerobic incubation of [2-^{14}C]glyoxylate (10 μC/μmole) in the assay system described, followed by chromatography of

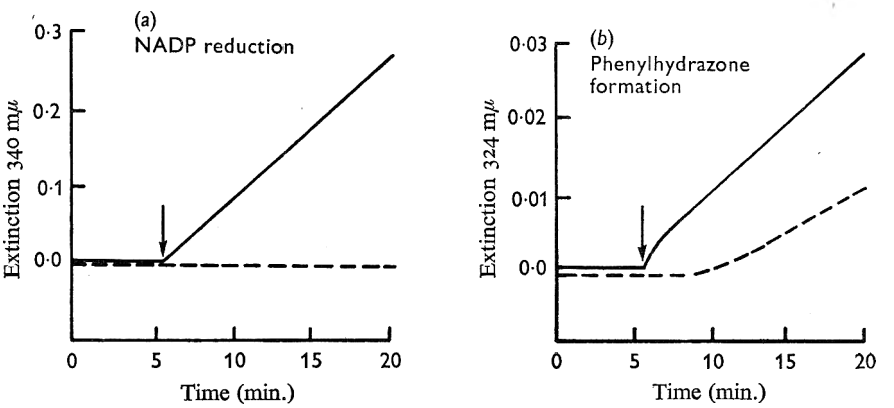

Fig. 3. Relative isocitrate dehydrogenase and isocitrate lyase activities of the yellow and blue regions of the ultracentrifuged extract of *Anacystis nidulans*. The assays for isocitrate dehydrogenase activity and isocitrate lyase activity were done as described in Methods. (*a*) NADP reduction. Isocitrate added at arrow to assay mixtures containing samples (equiv. 1·5 mg. protein) of the blue region (———); or samples (equiv. 0·28 mg. protein) yellow region (- - -). (*b*) Phenylhydrazone formation. Isocitrate added at the arrow to assay mixtures containing samples (equiv. 1·5 mg. protein) of the blue region (———); or samples (equiv. 0·28 mg. protein) of the yellow region (- - -).

the products, showed the major product to be malate with a smaller amount of citrate. Anaerobic conditions were used in this confirmatory experiment to minimize any subsequent metabolism of the isotopically-labelled malate. Extracts were prepared from *A. variabilis* and *A. nidulans* after growth in the presence or absence of sodium acetate (20 mM) and no difference in isocitrate lyase or malate synthase activity was found (Table 3). Very similar degrees of activity were present in extracts from either organism.

Two enzymes of the tricarboxylic acid cycle were also determined in extracts prepared from cultures grown in the presence or absence of acetate. These enzymes also showed no alteration in activity after growth with acetate (Table 4). The constant activity of citrate synthase is perhaps particularly interesting, serving as it does as the major entry point of acetate into the tricarboxylic cycle. The marked difference in activity of citrate synthase in extracts of *Anabaena variabilis* and *Anacystis nidulans* might be the result of procedural variations or species differences. The method of estimation used with *A. nidulans* involved the use of 5,5′-dithiobis-[2-nitrobenzoic acid] which we found to inhibit the analogous enzyme, malate synthase.

The blue-green algae examined did not adjust the enzymes metabolizing acetate when grown in the presence of sodium acetate. Preliminary results indicate that ribulose diphosphate carboxylase activity, the key enzyme of photosynthetic CO_2 fixation, was slightly altered after growth in the presence of sodium acetate. The growth rate of *Anabaena variabilis* was decreased to about half the control value by

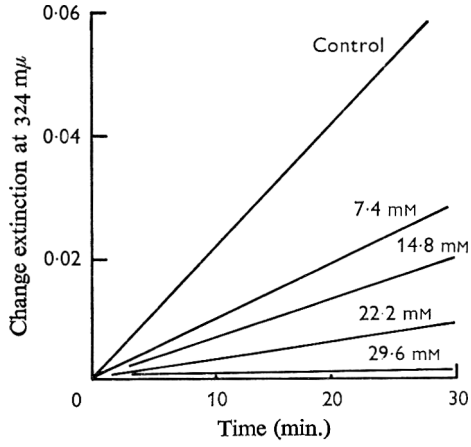

Fig. 3

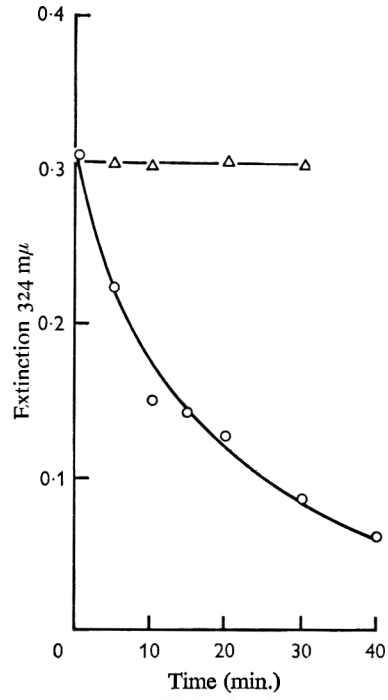

Fig. 4

Fig. 4. Inhibition of isocitrate lyase activity in extracts of *Anabaena variabilis* by sodium succinate. The assay was done as described in Methods and sodium succinate added at the concentrations indicated. The assay mixture contained equiv. 0·68 mg. protein in a total volume of 2·7 ml.

Fig. 5. Disappearance of glyoxylate in the presence and absence of coenzyme A. The assay for malate synthase activity in *Anacystis nidulans* was done as described. The residual glyoxylate was measured as its phenylhydrazone at 324 mμ after incubation with Dowex-treated cell-free extract (containing 3·2 mg. protein). The assay was done in the presence (—○—○—) and the absence (—△—△—) of added coenzyme A.

Table 3. *Glyoxylate cycle in the blue-green algae Anabaena variabilis and Anacystis nidulans*

Enzyme activities expressed in mμmoles/min./mg. protein and are the mean of several determinations.

Organism	Growth in presence 20 mM acetate	Isocitrate lyase	Malate synthase
A. variabilis	+	0·38	0·82
	−	0·39	0·80
A. nidulans	+	0·45	1·07
	−	0·43	1·00

interposing neutral filters between the culture vessel and light source, thus decreasing the available illumination. Under these conditions of light limited growth, [U-^{14}C]acetate (20 mM) was incorporated to the same extent as in the control and the activities of acetate kinase, isocitrate lyase and isocitrate dehydrogenase were unaltered (Table 5).

Table 4. *Activities of two tricarboxylic acid cycle enzymes*

Enzyme activities expressed in nμmoles/min./mg. protein and are the average of several determinations.

Organism	Growth in presence 20 mM acetate	Isocitrate dehydrogenase	Citrate synthase
Anabaena variabilis	+	5·0	6·1*
	−	4·7	5·7*
Anacystis nidulans	+	16·7	0·67†
	−	16·5	0·70†

* Procedure of Ochoa, (1955); † Procedure of Srere, Brazil & Gonen, (1963).

Table 5. *Anabaena variabilis: growth and enzyme activities under light-limiting conditions*

Enzyme activities expressed as mμmoles/min./mg. protein.

Conditions of growth	Growth rate (%)	% dry weight of algae derived from acetate	Acetate kinase activity	Isocitrate lyase activity	Isocitrate dehydrogenase activity
			(mμmoles/min./mg. protein)		
Full light	100	16·9	4·0	0·35	5·0
Limited light	48·7	15·7	4·1	0·40	4·8

DISCUSSION

The general autotrophic nature of blue-green algae has been known for many years and the occasional early reports of heterotrophic growth of certain species are often considered to be a result of impure cultures or incorrect classification. Allen (1952), in a detailed examination of the growth requirements of thirty blue-green algae, concluded that one species did require organic compounds in the growth medium, and the growth rate of several other species was improved in their presence. It appeared likely that these compounds were not serving as nutrients in themselves, but assisted in the absorption of certain mineral elements. Kratz & Myers (1955*b*), using three species of blue-green algae (including *Anabaena variabilis* and *Anacystis nidulans*), showed that there was only a limited respiratory response to a wide range of organic substrates. These workers postulated that a permeability barrier limited the ready availability of exogenous substrates to these organisms, a suggestion that had already been applied to other autotrophic micro-organisms. Studies in our laboratory showed that four species of blue-green algae were permeable to dichlorophenol-indophenol (Carr & Hallaway, 1965), although it is appreciated that permeability of microbial cell membranes is a highly selective phenomenon and it is quite possible for an organism to permit entry of a complex dye molecule and still be impermeable

to small organic substrates. The metabolism of acetate by blue-green algae appeared to be a fruitful level at which to consider the apparent autotrophic nature of the micro-organisms studied.

The data presented confirms earlier reports that acetate does not increase the growth or respiratory rate of the blue-green algae examined. However, experiments with [U-^{14}C]acetate indicates that incorporation occurs and that acetate contributes a significant fraction of the total dry cell weight. Similarly respiration of [1-^{14}C] and [2-^{14}C]sodium acetate was demonstrated, the proportion of [^{14}C] CO_2 released from each position being compatible with oxidation via the tricarboxylic acid cycle. It is evident that *Anabaena variabilis* and *Anacystis nidulans* are permeable to sodium acetate and possess enzymes necessary for its metabolism. The presence or absence of acetate in the growth medium did not alter the activities of acetate activating, glyoxylate cycle or two tricarboxylic acid cycle enzymes (Tables 2, 3, 4). The levels of isocitrate lyase in both *A. variabilis* and *A. nidulans* are low compared to those in other micro-organisms; this may indicate that another route of acetyl-CoA metabolism is operative. Following the incorporation of [^{14}C]acetate, Hoare & Moore (1965) did not find evidence of glyoxylate cycle operation in *A. nidulans*.

These results are in contrast to those obtained in many other microbial species where the inclusion of acetate in the growth medium increases the activity of enzymes concerned in its metabolism. The adaptive nature of isocitrate lyase has been shown in a large number of micro-organisms (see Kornberg & Elsden, 1961). The activity of this enzyme increased after growth in the presence of acetate. Likewise, in the green alga, *Chlorella vulgaris* isocitrate lyase and malate synthase increase in activity several fold after growth in the presence of acetate (Syrett, Merrett & Bocks, 1963). Perhaps the most pertinent comparison of the results presented is with the levels of enzyme activities in the facultative autotroph, *Hydrogenomonas* sp. strain H 165^{+}, after growth on acetate, or on H_2 and CO_2 (Trüper, 1965). All the enzymes examined in the blue-green algae here increased at least several fold after growth of Hydrogenomonas on acetate as compared with CO_2 and H_2. Isocitrate lyase activity was fifteen-fold higher, and acetyl-CoA synthetase six-fold higher after growth on acetate. When Hydrogenomonas was grown on fructose each of the enzymes discussed were significantly less active than after growth on acetate.

The failure to detect evidence of enzyme de-repression in blue-green algae, when acetate is added to the growth medium is unlikely to be due to a permanent repressing effect of CO_2, since replacement of air + CO_2 (95 + 5 v/v) mixture by air alone did not cause any increase in enzyme activity, However, if all the CO_2 in the gas phase was removed the culture died, so this point could not be established unequivocally. Similar attempts were made to induce increased enzyme activity in the presence of acetate by growing *Anabaena variabilis* under light-limiting conditions, which presumably reduced the pools of ATP and other high-energy phosphate esters to a minimum, but again there was no increase in the enzyme activities measured. It may be suggested that the lack of response exhibited to acetate, and other organic substrates, by blue-green algae could be due to a failure to adjust enzyme complement to environmental change.

Recently the utilization of carbon containing molecules other than CO_2 has been demonstrated in hitherto 'autotrophic' bacteria, *Nitrobacter agilis* (Ida & Alexander, 1965), *Thiobacillus thiooxidans* (Butler & Umbreit, 1966). It is evident that some

species of both blue-green algae and bacteria previously considered capable of using only CO_2 as a source of cell material can, at least under certain circumstances, use some organic molecules.

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Incorporation of ^{14}C-labelled Components of *Escherichia coli* and of Amino Acids by *Isotricha intestinalis* and *Isotricha prostoma* from the Sheep Rumen

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SUMMARY

Studies on the activities of suspensions of *Isotricha intestinalis* and *I. prostoma* freshly isolated from sheep rumen and washed almost free from bacteria were made by using [^{14}C]-amino acids and ^{14}C-labelled *Escherichia coli*. Bacteria were taken up by the protozoa at an approximately linear rate of up to 3000 bacteria/protozoon/hr for 24 hr, after which time at least 40% of the bacterial carbon was no longer in the form of whole bacteria. Amino acids supplied to the protozoa, either free in the medium or as intact bacteria, were incorporated unchanged into protozoal protein. Addition of individual free ^{12}C-amino acids to the medium partially inhibited incorporation into protozoal protein of ^{14}C from *Escherichia coli* labelled with the ^{14}C form of the same amino acid.

INTRODUCTION

Although some species of entodiniomorphid protozoa from the rumen have been cultured in the laboratory for long periods (e.g. Coleman, 1958, 1960, for *Entodinium caudatum*) the holotrichs have been cultured (Clarke & Hungate, 1966) for only a few months, because of the very laborious techniques necessary. The presence of bacteria appeared to be necessary for the maintenance of the rumen holotrich protozoa *Isotricha intestinalis* and *I. prostoma* and *Dasytricha ruminantium* and it has been suggested that one function of the bacteria may be the maintenance of a low redox potential in the medium (Gutierrez, 1958; Hungate, 1955; Clarke & Hungate, 1966). However, the role of the bacteria has not been investigated in detail. Studies on the carbohydrate metabolism of these holotrich protozoa have been made by using suspensions, freshly isolated from crude rumen fluid, which were more or less free from bacteria (Gutierrez, 1955, 1958; Heald, Oxford & Sugden, 1952). The protozoa maintained their fermentative ability and motility for some days under suitable conditions. Although *E. caudatum* has been shown to engulf bacteria and utilize bacterial amino acids (Coleman, 1964), there is little in the literature about bacterial feeding by holotrich protozoa or the amino acid metabolism of these protozoa. Gutierrez (1958) showed that *I. prostoma* engulfed bacteria but he did not investigate the fate of their cellular constituents. Harmeyer (1965) showed that mixed *I. intestinalis*

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and *I. prostoma* incorporated ^{14}C from $^{14}CO_2$ into protozoal aspartic acid, alanine, threonine, histidine and glutamic acid, but he did not investigate further the source of protozoal amino acids. The present paper reports studies on the uptake of a single bacterial species, namely *Escherichia coli*, and its metabolism by freshly isolated suspensions of *I. intestinalis* and *I. prostoma*.

METHODS

Isolation of Isotricha intestinalis and I. prostoma. Suspensions of these protozoa were obtained by a method based on those described by Heald *et al.* (1952) and Gutierrez (1955). Rumen fluid was obtained, 1 hr after feeding, from fistulated Clun Forest sheep on a diet of hay and oats, and was strained through muslin to remove large particles. Glucose was then added (to 0·5 %) and the fluid placed in a conical separating funnel at 39° for 60 min. Rapid synthesis of starch led to an increased specific gravity of the protozoa. The white layer which collected at the bottom of the funnel was run off into an 8-inch boiling tube containing 40 ml. of a 'salts solution' containing: (%) NaCl, 0·5; KH_2PO_4, 0·1; $CaCl_2$, 0·01; $MgSO_4$, 0·01; $NaHCO_3$ 0·5. This solution was steamed in an autoclave for 15 min. to remove dissolved air, then cooled and gassed for 2 min. with CO_2 before use. The tube was closed with a rubber bung and, after most of the protozoa had settled, the supernantant fluid was sucked off and replaced with a further 40 ml. salts solution. This procedure was repeated 4 times giving a final pellet of protozoa, over 95 % of which were *Isotricha intestinalis* and *I. prostoma* and less than 5 % *Polyplastron* spp. The protozoa were resuspended in salts solution (10 ml./500 ml. original rumen fluid) and used to inoculate experimental tubes.

Numbers of protozoa were estimated by fixing samples with 2·5 % (w/v) formaldehyde and diluting, when necessary, with salts solution. The number of protozoa present in 0·1 ml. samples of the suspensions was determined by scanning microscopically the whole area under a cover glass. At least 3 counts were made on each sample, a minimum of 600 protozoa being counted.

Incubation of protozoa. Incubation tubes (8 cm. × 0·8 cm., closed with polythene stoppers) contained about 2×10^4 protozoa in 2 ml. salts solution + 0·01 % (w/v) cysteine HCl. Additional amino acids, bacteria, penicillin or neomycin were added as required and tubes were gassed for 30 sec. with CO_2. Incubation was done with the tubes in a horizontal position at 39° unless otherwise indicated. Although actively motile, the protozoa tended to remain near the bottom of the tube. In the absence of antibiotics, less than 10^5 rumen bacteria/ml. were present at the beginning of the incubation.

After incubation, tubes were cooled to 0° and the protozoa were sedimented by centrifugation for 1 min. at low speed in an angle-head centrifuge (accelerating from 0 to 250 *g* during the 1 min. period). The protozoal pellet was washed 3 times by repeated resuspension in 2 ml. volumes of cold salts solution and centrifugation. The *Escherichia coli* when added could be quantitatively recovered from the supernatant fluid by centrifugation at 7000 *g* for 30 min. This pellet after resuspension and washing twice on the centrifuge in salts solution was the 'bacterial fraction'.

Preparation of Escherichia coli suspensions. Escherichia coli organisms, uniformly labelled with ^{14}C, were prepared by growth in C medium (Roberts *et al.* 1955) con-

taining 2 mg. and 1·6 μC [U-^{14}C]glucose/ml. Incubation was for 16 hr at 39° with a current of air bubbling through the suspension. To obtain *E. coli* labelled with a single ^{14}C amino acid, organisms were grown in C medium (Roberts *et al.* 1955) containing 0·2% glucose, the appropriate ^{14}C amino acid (0·8 μC + 10 μg./ml.) and 0·5 mg./ml. of the ^{12}C form (0·08 mg./ml. for [^{12}C]threonine) of those amino acids (if any) shown by Roberts *et al.* (1955) or by experience to be derived from the ^{14}C amino acid provided. The bacteria were harvested by centrifugation, washed twice in salts solution and resuspended in this solution before addition to incubation tubes. Approximately 10^9 *E. coli* organisms/ml. were present in each tube, unless otherwise indicated.

Bacterial colony counts. Numbers of viable rumen bacteria present in suspensions were determined by the method of Coleman (1962) with medium C. Colony counts of *Escherichia coli* were done on solid (2% agar) C medium (Roberts *et al.* 1955) + 2 mg. glucose/ml., by the technique of Miles & Misra (1938).

Fractionation of protozoa and Escherichia coli. Protozoa suspended in distilled water were broken for 30 sec. in a Potter homogenizer (Potter & Elvehjem, 1936) and were separated into a broken-protozoa pellet (BPP) and a broken-protozoa supernatant fluid (BPS) fraction by centrifugation for 30 min. at 7000 *g* (4°). Chemical fractionation of protozoa and *Escherichia coli* was done as described by Roberts *et al.* (1955). The protein fractions were hydrolysed with 6 N-HCl for 16 hr at 105° in a sealed tube. The tube was then cooled, opened and the acid removed on a boiling-water bath in a current of air. The amino acids were separated by two-dimensional chromatography (*sec*-butanol + formic acid + water; 70 + 10 + 20, by vol., 1st dimension; phenol + 0·880 sp.gv. ammonia + water, 80 + 0·3 + 20, w/v/v, second dimension). Radioactive amino acid spots were located by radioautography, cut out and the amino acids eluted with distilled water for determination of the total radioactivity present. α-Amino-nitrogen in the eluates was determined by the method of Cocking & Yemm (1954).

Radioactivity determinations. Samples were pipetted onto circles of lens tissue on aluminium planchets (2·5 cm. diam.). One drop 0·5% cetyltrimethylammonium bromide and 1 drop 0·2% polyvinyl alcohol were added before drying. Samples were counted with a thin mica end-window Geiger–Muller tube or a Nuclear Chicago gas flow counter and over 1000 counts recorded. Counts were corrected to infinite thinness.

Materials. [U-^{14}C]Glucose, [U-^{14}C] glycine, [U-^{14}C]L-leucine, [U-^{14}C]L-isoleucine, [U-^{14}C]L-serine, [U-^{14}C]L-alanine, [U-^{14}C]L-aspartic acid, [U-^{14}C]L-glutamic acid, [Me-^{14}C]L-methionine and [U-^{14}C]L-lysine were obtained from the Radiochemical Centre, Amersham, England. Benzylpenicillin and neomycin sulphate were obtained from Glaxo Laboratories, Greenford, Middlesex, England.

RESULTS

Incorporation of radioactivity from uniformly labelled Escherichia coli

The method used to study the uptake of bacteria by *Isotricha intestinalis* and *I. prostoma* was to measure the incorporation of ^{14}C from ^{14}C labelled *Escherichia coli* of known specific activity. The bacteria were separated from the protozoa by differential centrifugation as described in Methods. Under these conditions less than 0·5%

of the ^{14}C in the ^{14}C labelled *E. coli* was incorporated into the 'protozoal fraction' initially, after incubation at 0° for 24 hr or in the absence of protozoa.

At 39°, ^{14}C from uniformly labelled *Escherichia coli* was incorporated into the protozoal fraction at an approximately linear rate for at least 24 hr. This method of measuring bacterial uptake may underestimate the number of bacteria taken up, because as will be shown below, about 25% of the carbon in these bacteria may be liberated into the medium in a soluble form. Alternatively, the method could overestimate bacterial uptake if some digestion of the bacteria occurred outside the protozoa, followed by

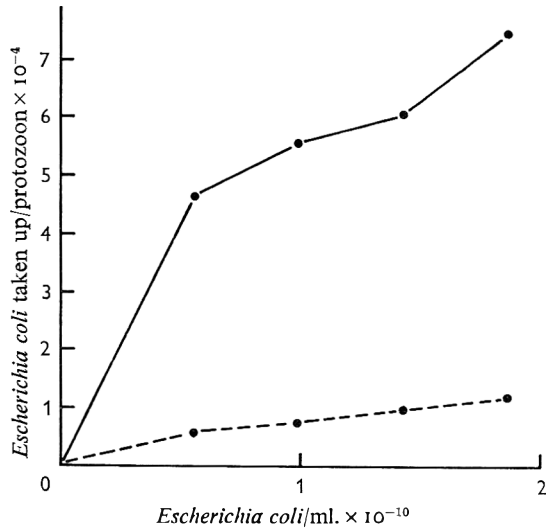

Fig. 1. Effect of *Escherichia coli* population density on uptake by $8{\cdot}5 \times 10^3$ protozoa incubated in 2 ml. salts solution + 0·01 % L-cysteine. The number of bacteria taken up was calculated from the ^{14}C in the protozoal fraction at 5 hr (- - -) and 24 hr (———) after incubation of protozoa with ^{14}C labelled *Escherichia coli* of known specific activity.

uptake of the liberated low molecular weight compounds. However, since in experiments on the incorporation of free amino acids less than 3% of total amino acid present was taken up in 24 hr, the error introduced thereby would probably be small. Previous heating of the *E. coli* to 75° for 10 min. (rendering them non-viable) did not decrease the rate of uptake. Incorporation increased with *E. coli* concentration at least up to $1{\cdot}8 \times 10^{10}$ *E. coli* organisms/ml., when the rate was equivalent to 3000 *E. coli*/protozoon/hr (Fig. 1), as compared with a rate of 12,000 bacteria/protozoon/hr for *Entodinium caudatum* (Coleman, 1964). In nine simultaneous determinations of incorporation over a 24 hr period, the coefficient of variation of the mean uptake was 4·6%, indicating that the sampling of the large dense protozoa was satisfactory.

Addition of 5% (v/v) autoclaved rumen fluid (ARF, Coleman, 1962), previously clarified by centrifugation at 7000 *g* for 30 min. did not affect incorporation of radioactivity from *Escherichia coli*. 10 or 20% (v/v) ARF in 24 hr inhibited by 25 or 40%, respectively. When tubes were gassed with 95% (v/v) N_2 + 5% (v/v) CO_2 instead of pure CO_2 (the concentration of $NaHCO_3$ being adjusted to maintain the same pH value), the protozoa were considerably less active after incubation for 24 hr and incorporation of radioactivity from *E. coli* was decreased by about 50%.

To determine whether the bacteria taken up by the protozoa were still viable, the following experiment was done. For 24 hr, 2×10^4 protozoa were allowed to take up ^{14}C labelled *Escherichia coli* from a suspension that contained 1×10^9 bacteria/ml. At intervals during the experiment, determinations were made of the ^{14}C in the protozoa and of the number of viable bacteria (colony count) in the medium, in washed intact protozoa, and in washed protozoa broken in a Potter homogenizer. At 4 and 24 hr, respectively, each protozoon contained ^{14}C from 1700 and 11,000 bacteria (as determined from ^{14}C in the protozoa), but there were only 110 and 0 viable bacteria inside each protozoon (as determined from the difference in colony count between intact and broken protozoa). Viability of *E. coli* incubated under the same conditions in the absence of protozoa fell by 50 % in 24 hr. These results show that *E. coli* were rapidly rendered non-viable after uptake by the protozoa.

After incubation for 24 hr of protozoa with uniformly ^{14}C labelled *Escherichia coli*, 19 % of the ^{14}C was found in the protozoal fraction, 20 % in the 7000 *g* supernatant fluid and 61 % in the bacterial fraction. 13 % of the ^{14}C was found in the supernatant fluid in the absence of protozoa, presumably the result of lysis of bacteria. The appearance of ^{14}C in the medium in the presence of protozoa might result from digestion of the bacteria and release of soluble products.

Table 1. *Distribution of radioactivity among fractions of sheep rumen protozoa (Isotricha species) and Escherichia coli*

Protozoa were incubated in the presence of 10^9 uniformly labelled *Escherichia coli* organisms/ml. for 24 hr before harvesting, washing, homogenising and separation into supernatant fluid (BPS) and protozoal pellet (BPP). The distribution of radioactivity among the chemical fractions was determined as shown in the text.

	Protozoa		*Escherichia coli*	
	BPP	BPS	Before incubation	After incubation
	Radioactivity (counts/min.)			
	5,330	3,640	45,100	39,600
	Fractionation (% total counts/min./fraction)			
Fraction				
Pool (cold TCA*)	1·5	10·4	6·4	2·7
Lipid	16·4	3·5	7·2	6·3
Ethanol-soluble protein	22·3	15·8	4·5	5·0
Nucleic acid	4·4	9·4	17·4	16·7
Residual pellet	43·9	50·2	44·5	50·3

* TCA = trichloroacetic acid.

Uniformly ^{14}C labelled *Escherichia coli* (before and after incubation) and the broken-protozoa pellet and broken-protozoa supernatant fluid fractions which had been incubated for 24 hr with uniformly ^{14}C labelled *E. coli* were fractionated (Table 1). 40 % of the total radioactivity incorporated into the protozoa was recovered in the broken-protozoa supernatant fluid. Since any whole bacteria present would have been recovered in the broken-protozoa pellet, this result shows that at least this proportion of the bacteria which were taken up had been disrupted. A higher proportion of the radioactivity was found in the ethanol-soluble protein and a lower proportion in the

nucleic acid fractions in both the broken-protozoa supernatant fluid and protozoal pellet than in *E. coli*. The amount of radioactivity in the amino acids of the residual protein fractions from the broken protozoa supernatant fluid and *E. coli* were compared. The total radioactivity present in each amino acid spot after two-dimensional chromatography of the hydrolysed protein in broken-protozoa supernatant fluid relative to that in glutamic acid (= 100) was as follows (results for *E. coli* are given in parentheses): alanine 6 (53); arginine 44 (39); aspartic acid 65 (93); glutamic acid 100 (100); glycine 17 (29); leucine + isoleucine + phenylalanine 196 (199); lysine 104 (62); methionine + valine 44 (68); proline 29 (41); serine 24 (31); threonine 30 (32); tyrosine 38 (29). It is noticeable that the alanine in the protozoa contained very little radioactivity although amino acid determinations confirmed the results of Harmeyer (1963) that the protozoal protein hydrolysate was not low in alanine. Some differences in the distribution of radioactivity in the other amino acids can also be seen in Table 1. If radioactivity in the protozoa was present only in whole *E. coli* the distribution of radioactivity among the amino acids would be expected to be the same in both protozoa and bacteria. This is therefore further evidence that the bacteria were broken down by the protozoa.

Incorporation of amino acids by protozoa

Incorporation of ^{14}C amino acids provided either free in the medium or in the form of *Escherichia coli* labelled with specific amino acids was investigated. Free ^{14}C amino acids were tested at 100 μg./ml. in the presence or absence of a mixture of the other 17 ^{12}C amino acids. The full 18 amino acid mixture contained glycine, L-aspartic acid, L-glutamic acid, L-arginine, L-methionine, L-lysine, L-threonine, L-proline, L-tryptophan, L-tyrosine, L-cysteine, L-serine, L-phenylalanine, L-histidine, L-valine, L-alanine, L-leucine, L-isoleucine, each at final concentration 100 μg./ml. To determine whether the amino acids were taken up by the protozoa directly or after incorporation into any bacteria present, the incubations were carried out in the presence or absence of 100 μg. benzylpenicillin + 40 μg. neomycin sulphate/ml.; 0 and $1-2 \times 10^6$ viable bacteria/ml., respectively, were found after 24 hr. These antibiotics did not affect the motility of the protozoa or their rate of uptake of radioactivity from ^{14}C-labelled *Escherichia coli*. Incorporation of each ^{14}C amino acid, tested alone was (μg./24 hr/2×10^4 protozoa): glycine 4·6 (5·2); leucine 4·0 (4·4); isoleucine 4·4; serine 3·7; methionine 1·7 (1·8); lysine 0·95 (1·6); aspartic acid 0·11; glutamic acid 0·08 (2·1); alanine 0·07 (0·08). The figures given in parentheses are values found in the absence of benzylpenicillin + neomycin and show that killing of bacteria by these antibiotics did not decrease the rate of uptake of most amino acids by the protozoa. Incorporation was not significantly affected by the presence of other amino acids and took place at a constant rate during the 24 hr incubation period. After chemical fractionation of protozoa which had incorporated glycine, leucine, serine, methionine or lysine, over 75% of the recovered radioactivity was found in the protein fractions in each case, with some in the cold trichloroacetic acid-soluble pool. Radioautography of chromatograms of the hydrolysed protein fractions indicated that no major interconversion of amino acids took place, although the possibility of leucine-isoleucine-phenylalanine, serine-glycine and lysine-arginine interconversions could not be ruled out because of incomplete resolution of these amino acids. The ^{14}C amino acids provided were the only radioactive compounds detected in the medium after removal of the protozoa.

From the results with uniformly-labelled *Escherichia coli*, it was apparent that bacterial carbon was incorporated into protozoal material; but these experiments provided no information on the metabolic fate of individual bacterial amino acids. As it was possible that the metabolism of free and bacterial amino acids might be different, samples of *E. coli* labelled singly with ^{14}C leucine, isoleucine, alanine, glutamic acid or lysine were prepared and the incorporation of radioactivity into the broken protozoa supernatant fluid fraction studied. Very low degrees of incorporation from ^{14}C alanine-labelled *E. coli* were found. Over 70% of the ^{14}C in the broken protozoa supernatant fluid was present in the protein, analysis of which showed that no amino acid was labelled in the protozoal protein which was not labelled in the bacteria fed. Unfortunately, under the conditions used, it was not possible to prepare *E. coli* which contained ^{14}C only in aspartic acid, glycine or serine, and some ^{14}C was found in other amino acids. After incubation of *E. coli* labelled principally with aspartic acid, glycine or serine, the majority of the ^{14}C was present as the same amino acid in the protozoa. It was not possible to eliminate amino acid conversions in the protozoa similar to those which had already occurred in the bacteria.

Incorporation of radioactivity from *Escherichia coli* labelled with specific ^{14}C amino acids was inhibited as follows by the addition to the medium of 500 μg./ml. of the ^{12}C form of that amino acid: leucine 69%, glycine 40%, isoleucine 81%, alanine 37%, lysine 38%, serine 5%, aspartic acid (−16%). To show that this was not a general effect of free amino acids on the rate of uptake of *E. coli* by the protozoa, it was shown that single free amino acids did not decrease the incorporation of ^{14}C from uniformly ^{14}C-labelled *E. coli* into protozoa and that [^{12}C]L-leucine did not decrease the incorporation of ^{14}C from *E. coli* labelled with [^{14}C]isoleucine. Addition of 100 μg. [^{12}C]L-leucine/ml. to incubations of protozoa with *E. coli* labelled with [^{14}C]leucine decreased incorporation of ^{14}C into the protozoal fraction to the same extent as it increased the radioactivity in the medium. The amount of ^{14}C present in the bacterial fraction was the same at the end of the incubation, whether in the presence or absence of [^{12}C]leucine. These results are consistent with the hypothesis that the protozoa digested at least part of the bacteria which they took up with the formation of free amino acids which were then incorporated into protein or released into the medium.

DISCUSSION

As far as the authors are aware, these studies represent the first quantitative demonstration of the uptake of bacteria by rumen holotrich protozoa and show that these protozoa are similar in this respect to *Entodinium caudatum* (Coleman, 1964). Gutierrez (1958) suggested that *Isotricha prostoma* selectively ingested certain rods from among many types of rumen bacteria, although several bacterial species were isolated from crushed protozoa. The present results show that *I. intestinalis* and *I. prostoma* took up a bacterium not isolated from the rumen, namely *Escherichia coli*, and then rapidly killed and digested this bacterium. As different bacterial species may have different survival times inside the protozoa, erroneous results may be obtained in attempts to determine, by examination of the bacteria in crushed protozoa, which bacterial species, if any, are important to the protozoa.

In a discussion of these initial results, obtained with the *in vitro* system described, two aspects of the biosynthetic metabolism of *Isotricha intestinalis* and *I. prostoma*

may be considered. One is whether these protozoa are capable of taking up bacteria and using bacterial components for synthesis of their own cellular materials. The second concerns the utilization of amino acids, either free or provided in bacterial form, for protein synthesis and the pathways which may be involved in interconversion of amino acids. Complete answers to these questions would obviously include valuable information about the biosynthetic pathways present in these protozoa.

It is clear that radioactivity from ^{14}C labelled *Escherichia coli* was taken up by the protozoa under the conditions described. Since 40% of the radioactivity from uniformly-labelled bacteria was found in the broken-protozoa supernatant fluid this could not have been in the form of whole *E. coli*. The differences in the distribution of ^{14}C between the chemical fractions of protozoa and bacteria (particularly among the amino acids of the protein fraction) after metabolism of [U-^{14}C]*E. coli* by the protozoa and also the apparent competition between single free ^{12}C amino acids and *E. coli* labelled with the ^{14}C form of that amino acid, support the hypothesis that bacterial protein was broken down and utilized for the synthesis of protozoal protein. Such an interpretation of the results would be greatly strengthened if a ^{14}C amino acid from *E. coli* could be demonstrated in an isolated specific protozoal protein. *Escherichia coli* was chosen for the present studies because of the wealth of information available about its metabolic pathways (e.g. Roberts *et al.* 1955). It would obviously be of considerable interest to compare incorporation of components of this bacterium with that of others, particularly those normally found in the rumen.

Amino acids provided either free or in *Escherichia coli* were incorporated into protein fractions of the protozoa, suggesting that some protein synthesis or turnover occurred under the conditions used. However, the ability of the protozoa to interconvert amino acids appeared to be limited, since only the ^{14}C amino acids provided were found to be labelled in protozoal protein fractions. It seems unlikely that incorporation of free amino acids by the protozoa occurred through initial incorporation by rumen bacteria followed by their ingestion by protozoa, since benzylpenicillin + neomycin eliminated bacterial growth but did not greatly affect amino acid incorporation.

Without a defined medium which supports growth of the holotrich protozoa of the rumen for prolonged periods, useful information may be still gained from studies of the activities of these organisms under the conditions described above. In particular, the utilization of bacterial components should be investigated further, in view of the observation that bacteria are essential for growth of these protozoa outside the rumen. However, as it is possible that biosynthetic pathways of the protozoa may not be the same *in vivo* as in these washed suspensions, the development of a growth medium for the organisms is of great importance in further understanding of their metabolism.

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Arginine Synthesis in *Proteus mirabilis*

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SUMMARY

Twenty-nine arginine auxotrophs of *Proteus mirabilis* strain 13 have been divided into eight groups which represent different biochemical blocks in the arginine biosynthetic pathway. The mutants were classified according to their growth requirements, syntrophism, accumulation of intermediates and enzyme deficiencies. The steps in the pathway are the same as those of *Escherichia coli* although mutants will not utilize *N*-α-acetyl-L-ornithine or *N*-acetyl-L-glutamate. However, these two substances were shown to be intermediates. Ornithine is synthesized via the linear route common to enterobacteria and not by transacetylation between *N*-α-acetyl-L-ornithine and L-glutamate as in some other bacteria and yeasts.

INTRODUCTION

Arginine synthesis has taxonomic significance as differences exist in the biosynthetic steps and enzymic control in various bacteria, yeasts and fungi (Vogel, 1953; Udaka, 1966; Smith, 1966). The arginine pathway as described for bacteria (Vogel, Bacon & Baich, 1963; Glansdorff, 1965; Udaka, 1966) is presented in Fig. 1. Some arginine

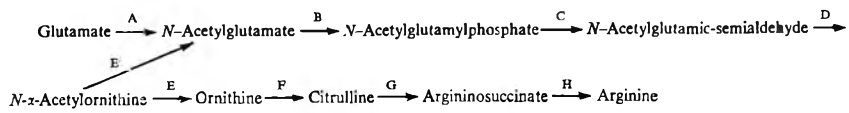

Fig. 1. Pathway of arginine synthesis in bacteria. A to H represent enzymic steps and the sites blocked in the corresponding *arg* mutants.

mutants of yeasts and fungi respond to proline (Bonner, 1946; Srb, Fincham & Bonner, 1950) whilst the early intermediates in the bacterial pathway are acetylated and distinct from the precursors in the proline pathway (Vogel, 1953; 1955). In *Micrococcus glutamicus* and some other bacteria transacetylation between L-glutamate and *N* α-acetyl-L-ornithine (Fig. 1, enzyme E′) occurs (Udaka & Kinoshita, 1958; Vogel & Vogel, 1963; Udaka, 1966; Hoare & Hoare, 1966). In these organisms the second enzyme in the pathway, *N*-acetyl-γ-glutamokinase (Fig. 1, enzyme B), is the site of feedback control, whereas in bacteria like *Escherichia coli* which lack this step the first enzyme, acetylglutamate synthetase (Fig. 1, enzyme A), is susceptible (Vyas & Maas, 1963; Udaka, 1966). The pathways of arginine biosynthesis in *E. coli* strains K12, W and B are identical (Gorini, Gundersen & Burger, 1961; Maas, 1961; Vogel, 1961) although mutants lacking *N*-acetylornithine-δ-transaminase (Fig. 1. enzyme D) have only been isolated in strain W (Vogel *et al.* 1963). Enzyme repression in *E. coli* B

also differs from the mechanism in strains K 12 and W (Gorini *et al.* 1961). The pathway of arginine synthesis in *Salmonella typhimurium* resembles that of *E. coli* (Demerec *et al.* 1960).

Baumberg, Bacon & Vogel (1965) concluded that in *Escherichia coli* K 12 the four clustered arginine genes, *arg E*, *C*, *B* and *H* have individual repressor recognition sites as repression of these genes was not affected in their mutant which has undergone a separation of these normally closely-linked genes. Vogel & Bacon (1966) have found that mutations tend to occur concomitantly in functionally-related although not closely linked arginine genes, and because of this they suggested that gene aggregates of such genes may be formed by chromosome folding. The finding of a genetic topography in *Proteus mirabilis* similar to that of *E. coli* would confirm the importance and generality of these observations regarding gene sequence and clustering. As a preliminary to genetic studies arginine synthesis in *P. mirabilis* was investigated.

METHODS

Media. The minimal medium was that of Grabow & Smit (1967), with agar (1·75 %, w/v) in solid media. When enriched with a mixture of growth factors (Novick & Maas, 1961) this medium was used as a complete defined arginine-free medium (AF), to which various concentrations of arginine could be added when required. The complete medium was Difco SS agar for *P. mirabilis* and Difco MacConkey agar for *E. coli* mutants. The broth was that of Coetzee & Sacks (1960*a*).

Chemicals. Amino acids, growth factors, arginine precursors, coenzymes and other chemicals were obtained from the Nutritional Biochemicals Corporation, Cleveland, Ohio, U.S.A.; Sigma Chemical Company, St Louis 18, Missouri, U.S.A.; British Drug Houses Ltd., Poole, England or Koch-Light Laboratories Ltd., Colnbrook, Buckinghamshire, England. *N*-α-Acetyl-L-ornithine was synthesized (Vogel, 1955) from *N*-δ-carbobenzoxy-L-ornithine obtained from Dr Theodor Schuchardt, G.M.B.H. & Co., München, Germany. *o*-Amino-benzaldehyde was synthesized from *o*-nitro-benzaldehyde by the method of Albrecht, Scher & Vogel (1962). Carbamyl phosphate was synthesized according to Jones, Spector & Lipmann (1955) and extemporate hydroxylamine was prepared from hydroxylammonium chloride (E. Merck A.G., Darmstadt, Germany) by the method of Beinert *et al.* (1953).

Bacteria. Argininеless mutants of *Proteus mirabilis* strain 13 and a streptomycin-resistant mutant of this organism, 13 *str-r* (Coetzee & Sacks, 1960*b*) were obtained (Prozesky & Coetzee, 1966) by treatment with manganese chloride and hydrogen peroxide (Demerec, Bertani & Flint, 1951). A penicillin enrichment technique (Lederberg & Zinder, 1948) was followed by replica plating (Lederberg & Lederberg, 1952). Some of the mutants were selected by the method of Gorini & Kaufman (1960). The nutritional requirements of mutants were determined auxanographically (Lederberg, 1946). Mutant *arg A* 1, *B* 1 with two blocks was obtained by transduction with phage 34/13 (Prozesky, in preparation). *E. coli* K 12 arginine mutants W 3422, W 3679, PA 201 were obtained from Dr W. K. Maas (Vyas & Maas, 1963). The *E. coli* K 12 arginine mutant GSB 1 was obtained from Dr N. Glansdorff (Glansdorff & Sand, 1965). A secondary *E. coli* mutant W 4422 P able to grow with acetylglutamate was obtained from mutant W 3422. It has the same phenotype as mutant W 3422 B of Vyas & Maas (1963) and was selected in the same way. The mutants and their phenotypes

are listed in Table 1. Cultures were maintained on nutrient agar slopes at 4° and the incubation temperature was 37°. Fluid cultures were aerated with sterile air.

Syntrophism. All the *Proteus mirabilis* and *Escherichia coli* mutants were tested with one another. Overnight broth cultures of mutants were streaked in parallel about

Table 1. *Phenotypes of arginineless mutants*

Mutants	Enzyme block	Reaction to streptomycin (1 mg./ml.)	Growth response with ac-glu	ac-orn	orn	cit	arg
Proteus mirabilis							
*arg A*1	A	r	−	−	+	+	+
*arg A*2	A	s	−	−	+	+	+
*arg A*3	A	s	−	−	+	+	+
*arg A*4	A	r	−	−	+	+	+
*arg A*5	A	s	−	−	+	+	+
*arg A*6	A	s	−	−	+	+	+
*arg A*1, *B*1	A+B	r	−	−	+	+	+
*arg B*1	B	s	−	−	+	+	+
*arg B*2	B	s	−	−	+	+	+
*arg C*1	C	s	−	−	+	+	+
*arg D*1	D	s	−	−	+	+	+
*arg D*2	D	s	−	−	+	+	+
*arg D*3	D	s	−	−	+	+	+
*arg D*4	D	s	−	−	+	+	+
*arg D*5	D	s	−	−	+	+	+
*arg D*6	D	s	−	−	+	+	+
*arg E*1	E	r	−	−	+	+	+
*arg E*2	E	s	−	−	+	+	+
*arg E*3	E	r	−	−	+	+	+
*arg F*1	F	r	−	−	−	+	+
*arg F*2	F	r	−	−	−	+	+
*arg F*3	F	r	−	−	−	+	+
*arg F*4	F	s	−	−	−	+	+
*arg F*5	F	s	−	−	−	+	+
*arg G*1	G	s	−	−	−	−	+
*arg G*2	G	s	−	−	−	−	+
*arg H*1	H	s	−	−	−	−	+
*arg H*2	H	s	−	−	−	−	+
*arg H*3	H	s	−	−	−	−	+
Escherichia coli							
*W*3422	A	s	−	+	+	+	+
*W*3422P	A	s	+	+	+	+	+
*W*3679	B	s	−	+	+	+	+
GSB1	C+E	r	−	−	+	+	+
PA201*	E	r	−	−	+	+	+

r = resistant; s = sensitive; ac-glu = acetylglutamate; ac-orn = acetylornithine; orn = ornithine; cit = citrulline; arg = arginine; + = growth; − = no growth.

* Also requires threonine, leucine, thiamine, histidine.

2 mm. apart on minimal agar enriched with 0·01 % Difco nutrient broth powder and incubated for 48 hr (Grabow & Smit, 1967). Results were confirmed by auxanography with isolated accumulated precursors obtained from a representative of each of the *P. mirabilis* mutant types (see below). These precursors were tested for growth response with all the *E. coli* mutants and a representative of each of the *P. mirabilis* mutant types.

Isolation of accumulated precursors. Cultures were grown in 1600 ml. minimal

medium containing L-arginine hydrochloride (8 μg./ml.). L-Citrulline or L-ornithine was substituted for arginine when possible to minimize possible effects of repression or feedback control (Gorini & Maas, 1958; Vogel, 1961). After incubation for 36 hr at 37° the cultures were Seitz-filtered and any argininosuccinate, citrulline, ornithine and acetylornithine which had accumulated in the medium was extracted using a column (25 × 3·5 cm.) of cation exchange resin (Amberlite IR 120, H^+ form). The column was washed with 500 ml. water and eluted with 150 ml. N-NH_4OH (Grabow & Smit, 1967). The eluates were vacuum-evaporated to dryness and the residues dissolved in 0·5 ml. water for preliminary chromatography. *N*-Acetylglutamic-γ-semialdehyde and acetylglutamate were concentrated and purified as described by Vogel (1953) and Vyas & Maas (1963) respectively.

Chromatography. A modification of the technique of Grabow & Smit (1967) had to be used because of the residual salts content of the sample. Duplicate one-dimensional chromatograms of samples and standards were developed for 16 hr on Whatman No. 3MM paper with *n*-butanol + pyridine + water (1 + 1 + 1, by vol.) as solvent by the descending technique. Standards were located by spraying with ninhydrin (0·2 %, w/v, in acetone) and heating at 100° for 5 min. Acetylglutamate was located with 0·05 % (w/v) bromcresol-green in 95 % ethanol (Vyas & Maas, 1963). Corresponding areas on duplicate chromatograms containing the samples were cut out, eluted with water and the eluates vacuum-evaporated to dryness. The residues were taken up in 0·1 ml. water and re-chromatographed on Whatman No. 1 paper in two dimensions. The solvents were: first dimension, *n*-butanol + formic acid + water (77 + 10 + 13 by vol.); second dimension, *n*-butanol + pyridine + water as above. Acetylglutamate gave elongated spots with these solvents when applied to chromatograms in sufficient quantities for clear definition with the bromcresol-green method. This precursor was therefore only chromatographed in one dimension with each solvent which is sufficient for identifying this substance (Vyas & Maas, 1963).

Precursor identification. Substances on chromatograms were identified by position and by mutant response. The mutant response technique was a modification of the method of Winsten & Eigen (1950). Minimal agar was seeded with about 10 ml. washed bacteria from an overnight broth culture of a mutant strain of *Escherichia coli* or *Proteus mirabilis* with a known growth response. The seeded medium was poured as plates of 30 × 30 × 1 cm. (Maré, Coetzee & de Klerk, 1964). Chromatograms were cut into squares, sterilized with ultraviolet light and placed on the agar for 5 min. After removal of the paper the plates were incubated for 48 hr and areas of growth were compared with stained chromatograms of samples and standards which had been developed concurrently. *N*-acetylglutamic-γ-semialdehyde was identified by its colour reaction with *o*-amino-benzaldehyde and by preparing its hydrazone derivative with 2,4-dinitro-phenylhydrazine (Vogel, 1953).

Enzymes. The enzymes and their assay methods are listed in Table 2. In general the specific activities of enzymes in cell-free extracts of all the auxotrophs were compared with those of the prototroph. However, the wild-type level for glutamate acetylation (enzyme A) was measured with resting cell suspensions of mutant *arg B* 1 (Vyas & Maas, 1963) and the mutant enzyme level was determined with the double mutant, *arg A* 1, *B* 1; acetylglutamate was assayed with *Escherichia coli* W 3422 P. To eliminate interference from acetylornithinase, transacetylation between acetylornithine and glutamate (enzyme E′) was measured in mutant *arg E* 1.

Table 2. *Enzymes of the arginine pathway of Proteus mirabilis*

Enzyme		E.C. no.	Position in pathway	Reaction product measured	Reference for method	Enzyme specific activity in prototroph (units)
Systematic name	Trivial name					
AcetylCoA: L-glutamate *N*-acetyltransferase	Acetylglutamate synthetase	2.3.1.1	A	*N*-Acetylglutamate	Vyas & Maas (1963): measured with mutant *arg B* 1	59·3*
ATP: *N*-acetylglutamate 5-phosphotransferase	*N*-Acetyl-γ-glutamokinase	—	B	*N*-Acetyl-γ-glutamyl hydroxamate	Baich & Vogel (1962) as used by Glansdorff (1965)	3·5†
α-*N*-Acetyl-L-glutamate-γ-semialdehyde: NADP oxidoreductase	*N*-Acetylglutamic-γ-semialdehyde dehydrogenase	1.2.1.4	C	NADPH	Baich & Vogel (1962; Glansdorff & Sand (1965)	0
L-Ornithine: 2-oxoacid aminotransferase	*N*-Acetylornithine-δ-transaminase	2.6.1.11	D	*N*-Acetyl-glutamic-γ-semialdehyde	Albrecht & Vogel (1964)	3·6‡
α-*N*-Acetyl-L-ornithine amidohydrolase	Acetylornithinase	3.5.1.d	E	Ornithine	Vogel & Bonner (1956)	22·7†
N-Acetylornithine; L-glutamate *N*-acetyl-transferase	Acetylornithine-glutamate transacetylase	—	É	Ornithine	Udaka (1966): measured with mutant *arg E* 1	0·05†
Carbamoylphosphate: L-ornithine carbamoyl-transferase	Ornithine transcarbamylase	2.1.3.3	F	Citrulline	Jones (1962)	3·1†
L-Citrulline: L-aspartate-ligase	Condensing enzyme	6.3.4.5	G	Disappearance of citrulline	Ratner (1955)	0·5†
L-Argininosuccinate-arginine lyase	Argininosuccinase	4.3.2.1	H	Ornithine	Baumberg *et al.* (1965)	1·0†

* 1 unit = 1 μmole/mg. protein/hr.
* 1 unit = 1 μg./mg. dry bacteria/hr.
‡ 1 unit = 0·1 increase in absorbancy at 440 mμ/mg. protein/hr.
NADPH = Reduced nicotinamide-adenine dinucleotide phosphate.
E.C. no. = Enzyme Commission number.

Preparation of cell-free extracts. Organisms (approximately 500 mg. dry wt) were harvested from 1600 ml. of medium AF supplemented with L-arginine hydrochloride (8 μg./ml.) after 36 hr. They were washed twice in the buffer recommended for the enzymic assay (Table 2) and resuspended in 25 ml. of the same buffer. Suspensions were disrupted in a Raytheon model S102A oscillator (9 kcyc./sec. for 45 min.) and centrifuged at 30,000 *g* for 30 min. in a Sorvall SS-4 centrifuge. The supernatant fluid was dialyzed against 2 l. of the corresponding buffer with 2 changes. The above manipulations were all performed at 4°. Protein content of the extracts was determined by the method of Gornall, Bardawill, & David (1949).

RESULTS

Arginineless auxotrophs. A total of 103 arginine-requiring mutants were obtained from 30 isolation experiments and a group of 29 mutants was chosen for investigation on the basis of one phenotype per isolation.

Growth requirements. The growth requirements of these mutants are given in Table 1, and three classes of *Proteus mirabilis* mutant can be distinguished. They responded to ornithine, citrulline or arginine and appear to be blocked in the synthesis of ornithine, the conversion of ornithine to citrulline or the conversion of citrulline to arginine, respectively (Fig. 1). None of the mutants responded to glutamate, acetylglutamate, acetylornithine, acetylglutamic-γ-semialdehyde, argininosuccinate or proline. No secondary mutants (Vyas & Maas, 1963) able to grow with acetylglutamate, acetylornithine, acetylglutamic-γ-semialdehyde or argininosuccinate were isolated after exposure to these intermediates, although the selection method proved satisfactory for the isolation of *Escherichia coli* W3422P from mutant W3422.

Syntrophism. The above results were confirmed by studies of syntrophism with all the mutants; the same three mutant classes were identified. Mutants subsequently defined as types *G* and *H* fed all other mutants but were fed by none. Type *F* mutants were fed by type *G* and *H* mutants and fed the class of mutants which included types *A*, *B*, *C*, *D*, *E*. This latter class of mutants did not feed the other classes of mutant nor was syntrophism exhibited between pairs of mutants within this class despite the fact that they accumulate precursors (see below). These mutants were further classified by syntrophism tests with well-characterized *A*, *B* and *E* mutants of *Escherichia coli*. *Proteus mirabilis* mutants types *F*, *G* and *H* fed all the *E. coli* mutants, while none of the *E. coli* mutants fed any of the *P. mirabilis* mutants. *Proteus mirabilis E* mutants fed *E. coli A* and *B* mutants but not *E* mutants. *Proteus mirabilis B* and *C* mutants only fed the secondary *E. coli* mutant of type *A* (W3422P which responds to acetylglutamate). *Proteus mirabilis A* and *D* mutants did not feed any *E. coli* mutants nor did the *P. mirabilis C* mutant feed the *E. coli* type *B* mutant. The results of syntrophism tests with the auxanographic technique gave identical results. Positive identification of the following groups of mutants was possible on the basis of growth response and syntrophism: $A+D$; $B+C$; E; F; $G+H$.

Accumulation of precursors. All the mutants were tested for accumulation of intermediates. Apart from group *arg A* mutants the other groups accumulated preceding precursors in the medium. Only in the case of *arg H* mutants was more than one accumulated precursor identified. The precursors, their R_F values, the mutants which excrete them and the mutants used for their identification are listed in Table 3. The

Table 3. *Precursors accumulated by argininеless mutants*

Proteus mirabilis and *Escherichia coli* mutant groups	Accumulated precursor	R_F value in solvent: Butanol-formic acid water	R_F value in solvent: Butanol pyridine-water	Mutants used for identification*: Positive growth response	Mutants used for identification*: Negative growth response
Arg A	None	—	—	—	—
Arg B, arg C	*N*-acetylglutamate	0·65	0·38	*E. coli* W 3422 P	*E. coli* W 3422
Arg D	*N*-acetylglutamic-γ-semialdehyde	†	†	—	—
Arg E	*N*-α-acetylornithine	0·04	0·06	*E. coli* W 3679	*E. coli* PA 201, *arg D* 1
Arg F	Ornithine	0·01	0·15	*E. coli* PA 201	*arg G* 1, *arg H* 1
Arg G, arg H	Citrulline	0·03	0·21	*arg F* 1	*arg G* 1, *arg H* 1
Arg H	Argininosuccinate	0·01	0·04	None	All classes

* *Proteus mirabilis* mutants except where noted.

† Chromatography not done. Identified by a colour reaction with *o*-aminobenzaldehyde and by preparing the hydrazone derivative (see 'methods').

results of the growth requirement experiments, syntrophism tests and accumulation studies made it possible to identify the following groups of mutants: *A*; *B*+*C*; *D*; *E*; *F*; *G*; *H*. Distinction between the *B* and *C* mutants could only be made by enzyme assays.

Enzyme studies. Seven enzymes in the pathway could be demonstrated in the wild-type organism (Table 2), while all the mutants in each of these seven mutant groups showed complete absence or only traces of the corresponding enzyme activity. The enzyme deficiencies in these mutants positively confirmed their classification based on the results of the preceding investigations. These results also made a distinction between the *arg B* and *arg C* mutants although *N*-acetylglutamic-γ-semialdehyde dehydrogenase (enzyme C) could not be demonstrated. Unsuccessful attempts were made to demonstrate it alone and also in combination with enzymes B or D as for *Escherichia coli* (Baich & Vogel, 1962; Glansdorff & Sand, 1965). Previous work with *Proteus mirabilis* strain 18 also yielded negative results (Smith, 1966). Failure to demonstrate enzyme C may be due to oxidation of reduced nicotinamide-adenine dinucleotide phosphate (Smith, 1966) and attempts to separate it from this activity were unsuccessful. However, mutant *arg C* 1 has the wild-type level of *N*-acetyl-γ-glutamokinase (enzyme B), activity while enzyme B activity is completely lacking in *arg B* mutants. The low level of acetylornithine-glutamate transacetylase (enzyme E′) and the high level of acetylornithinase (enzyme E) found indicate that the transacetylation reaction probably does not participate in the proteus pathway. The absence of any *arg E′* mutants is additional evidence for this conclusion.

DISCUSSION

The findings indicate that the route of arginine synthesis in *Proteus mirabilis* is identical to that found in *Escherichia coli* and *Salmonella typhimurium* (Demerec *et al.* 1960; Vogel *et al.* 1963; Baumberg *et al.* 1965). The proteus mutants differ from the *E. coli* mutants by their inability to grow with acetylornithine. However, it appears that this substance is an intermediate in the pathway because it is accumulated by *arg E* mutants and it is the substrate for acetylornithinase (enzyme E) which is present in *P. mirabilis*. The failure of mutants to respond to acetylglutamate is in accordance with similar findings in *S. typhimurium* (Demerec *et al.* 1960). Secondary mutants of *E. coli* are able to utilize acetylglutamate which may reflect the acquisition of permeability for this substrate; an acetylornithine permease has been identified in *E. coli* (Vogel, 1960). Consequently, it appears that *P. mirabilis* is cryptic (Cohen & Monod, 1957) for acetylornithine and acetylglutamate. Although some experimental conditions were different the specific activities for the enzymes in the arginine biosynthetic pathway (Table 2) are similar to those reported for *E. coli* (Vyas & Maas, 1963; Glansdorff & Sand, 1965; Vogel & Bonner, 1956; Udaka, 1966 and Gorini, 1963). The low specific activity of enzyme E′ (Table 3) indicates that transacetylation between acetylornithine and glutamate does not play an important part in the biosynthesis of arginine in *P. mirabilis*. Udaka (1966) encountered a similar situation with *P. vulgaris*. The inability to demonstrate *N*-acetylglutamic-γ-semialdehyde dehydrogenase activity in *P. mirabilis* does not exclude the existence of two steps between acetylglutamate and *N*-acetylglutamic-γ-semialdehyde (Baich & Vogel, 1962). There is strong evidence that reaction C (Fig. 1) exists: Mutant *arg C* 1 accumulates acetylglutamate and has

the wild-type level of *N*-acetyl-γ-glutamokinase (enzyme B) activity (enzyme B activity is completely lacking in *arg B* mutants). The observed syntrophism and accumulation of arginine precursors is in marked contrast with the absence of these eatures in the methionine pathway of *P. mirabilis* strain 13 (Grabow & Smit, 1967) which indicates that this is a peculiarity of the latter pathway in this organism and not a common property of *P. mirabilis*.

Linked transduction with some of these mutants has been described (Prozesky & Coetzee, 1966) and the genetic studies in progress may allow further comparison between arginine synthesis in *Escherichia coli* and *Proteus mirabilis*.

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Phase Variation of Flagellar Antigens in Salmonella: Abortive Transduction Studies

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SUMMARY

Motility was transduced by phage P22 to non-motile (*fla*$^-$, etc.) *Salmonella typhimurium* strains, and the flagellar antigens of abortive transductants inferred from the inhibition by antisera of the trails they produced in semi-solid medium. When the recipient had a *fla*$^-$ gene closely linked to *H1* (phase-1 flagellar antigen locus) nearly all the *fla*$^+$ abortive transductants evoked from a recipient culture in latent phase 1 manifested the phase-1 antigens of both recipient and donor, whereas those from a culture in latent phase 2 showed neither phase-1 antigen. Thus the expression of an exogenote *H1* allele, like that of the chromosomal *H1* allele, was regulated by the phase-determinant of the recipient, at or near *H2* (phase-2 flagellar antigen locus). An *H1* gene adjacent (*cis*) to *ah1*$^-$ (*H1* activator gene), in the chromosome or in the exogenote, was unexpressed in *ah1*$^+$ or *fla*$^+$ abortive transductants in phase 1. This suggests that *ah1*$^-$ mutants are *H1*-operator-negative mutants.

When the recipient was a phase-1, and therefore non-motile, culture of an *ah1*$^-$ or 'phase-1-curly' mutant, lysates of phase-2 cultures, but not of phase-1 cultures of the same donor, evoked trails, attributable to *H2* abortive transductants. They expressed the donor *H2* allele, but not the recipient *H2* allele—nor the previously expressed *H1* allele of the 'phase-1-curly' recipient. It is inferred that a phase-determinant regulates the expression of the *H2* gene adjacent (*cis*) to it (or of which it forms part) but not that of another *H2* gene in the same cell; and that it controls the expression of *H1* via a repressor substance, not via an inducer. The exceptional *H1* allele *H1-1,2* determines a flagellin of antigenic character *1,2*, apparently identical with that determined by a common *H2* allele. *H1-1,2* and the common *H1* allele *H1-b* were simultaneously expressed in *fla*$^+$ abortive transductants, which suggests that neither *H2* flagellin nor *H2* messenger RNA functions as the repressor of *H1*. It is proposed that an operon, comprising *H2* and the structural gene for an *H1* repressor substance, has alternative metastable states, 'on' in phase 2 and 'off' in phase 1, comparable to the wild-type state and to that of an operator-negative mutant.

INTRODUCTION

Most Salmonella species have two flagellar antigens, the phase-1 and phase-2 antigens, and show diphasic variation. A given bacterium manifests only one of these two antigens (and is said to be in the corresponding phase) and produces descendants most of which are in the same phase as itself, but amongst these a minority in the

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other phase arise by an apparently random mutation-like process. In *S. typhimurium* and *S. abony* the rate of change from phase 1 to phase 2 ranges from about 10^{-5} to 10^{-3} per bacterium per generation, and of the reverse change from about 10^{-4} to 5×10^{-3} (Stocker, 1949; Mäkelä, 1964). The various phase-1 and phase-2 antigens which occur in different species or serotypes are determined by a series of alleles at two loci termed, respectively, *H1* and *H2*, which are widely separated on the bacterial chromosome (Smith & Stocker, 1962; Sanderson & Demerec, 1965; Mäkelä, 1964). Probably *H1* and *H2* are the structural genes for the phase-1 and phase-2 flagellar proteins (flagellins) which constitute the flagella of phase-1 or phase-2 bacteria (McDonough, 1965). The production of flagella of either antigenic type is prevented by *fla* mutations, some of which are closely linked to, and co-transducible with, *H1*. Though *fla*$^-$ strains lack flagella, they undergo phase-variation, their (latent) flagellar antigenic phase being revealed by, for instance, the phase of their flagellate mutants (Stocker, Zinder & Lederberg, 1953). Mutation at another locus, *ah1*, closely linked to (or perhaps a part of) *H1* prevents the formation of phase-1 flagella—so that *ah1*$^-$ mutants alternate between a normal, flagellate, phase 2 and a phase 1 in which the bacteria are non-flagellate, and therefore non-motile (Iino, 1961). Another kind of mutation, within *H1* or very closely linked to it, produces the 'phase-1-curly' phenotype (Iino, 1962). Such mutants when in phase 2 produce normal flagella and are normally motile; but when in phase 1 they make functionally deficient flagella with an abnormally short wavelength ('curly').

In transductional crosses between Salmonella strains differing in phase-1 and phase-2 antigens, the phase of complete transductants shows that the phase of a bacterium is determined at (or close to) its *H2* locus. It seems that *H2* (or a phase-determinant locus, very closely linked to *H2*) exists in either of two 'states': when it is in the active state, the flagellin specified by *H2* is produced, that specified by *H1* is not produced and the cell is in phase 2; when *H2* is in the inactive state the flagellin specified by *H2* is not produced, that specified by *H1* is produced and the cell is in phase 1 (Lederberg & Iino, 1956). The dependence of the phase of a transductant (and so, presumably, of all other cells) on the 'state' of its *H2* locus is inferred from the observation that the phase of a transductant clone is always that of the parent strain (donor or recipient) from which it derived its *H2* allele. For instance, with an *H1*-linked *fla*$^-$ recipient all *fla*$^+$ transductants, whether or not they inherit the donor *H1* allele and whatever the phase of the donor, are found to be in phase 1 if the recipient culture was in (latent) phase 1, and in phase 2 if it was in (latent) phase 2. By contrast, transduction of an active *H2* locus from a donor in phase 2 to a motile recipient results in *H2* transductants which are in phase 2, regardless of the phase of the recipient—whereas if the donor is in phase 1, i.e. with an inactive *H2*, transduction of *H2* is undetectable, because the *H2* transductants, having acquired an inactive *H2*, are consequently in phase 1, and so do not express their new phase-2 antigen. Iino & Lederberg (1964) discussed the interpretation of phase variation and related phenomena in terms of current concepts of the regulation of genes which specify proteins. Among other possibilities they suggested that *ah1* might be an operator locus for the *H1* gene which it adjoins. They listed several possible explanations for the epistasis of *H2* to *H1*.

The main evidence for the above interpretation of phase-variation comes from analysis of the flagellar antigens of complete transductants, which are haploid for all loci concerned. Study of the flagellar antigens of cells heterozygous for the *H1* or *H2*

regions would permit examination of dominance and *cis*/*trans* effects, and allow a test of the ability of a chromosomal *H2* locus to regulate the expression of *H1* and *H2* genes not located in the same chromosome. We now describe an analysis of the flagellar antigens of such partial heterozygotes, obtained by the abortive transduction of the *H1* or *H2* regions. The abortive transduction of motility to non-motile recipient strains results in the appearance of trails, i.e. linear groups of microcolonies extending away from the site of inoculation, when the transduction mixture is incubated on semi-solid medium. Each trail marks the path of the successive descendants carrying a transduced fragment of donor chromosome, which is never replicated but which contains a gene conferring the motile phenotype (Stocker *et al.* 1953; Lederberg, 1956; Stocker, 1956). Antisera reacting with the flagella of a motile bacterium immobilize it, and the flagellar antigen(s) of trail-producing cells can be inferred from the ability of test sera, incorporated in the semi-solid medium, to prevent the formation of trails. Lederberg (1956) thus determined the flagellar antigens of abortive *fla*$^+$ transductants in a monophasic strain. We used diphasic donor and recipient strains. The recipient strains used were non-motile, either because they were *fla*$^-$ mutants, or because they were phase-1 cultures of strains which are non-motile when in this phase (*ah1*$^-$ or 'phase-1-curly'). Some such experiments are described elsewhere (Pearce & Stocker, 1965) as evidence that the chromosome fragments transduced by phage P22 are not, as formerly supposed, invariant in composition.

METHODS

Bacterial strains. The strains used were all *Salmonella typhimurium* and, except where the contrary is stated, were derivatives of strain LT2, most of them with various nutritional and drug-resistance mutations, not here listed because not relevant. In some, one or both of the wild-type flagellar antigen determinants of *S. typhimurium*, *H1-i* and *H2-1,2*, had been altered by mutation, or replaced by transduction.

The non-flagellate mutants used were *fla-56* and *fla-57*, of complementation group A, and *fla-58*, of complementation group D (Joys & Stocker, 1965). All three strains when treated with phage P22 grown on LT2 *fla*$^+$ produce many long trails, and their *fla* sites are closely linked to *H1* (rate of co-transduction of *H1* with *fla*$^+$ > 0·02 Joys & Stocker, 1965; Pearce & Stocker, 1965). These *fla* mutants were obtained in lines in which the wild-type phase-1 antigen, *i*, had been modified by mutation at *H1* (Joys & Stocker, 1966)—but the relatively minor serological differences between the modified and wild-type antigen are of no significance for the present purpose.

The *ah1*$^-$ strains used were two of the three non-identical, non-complementing *ah1*$^-$ strains described by Iino (1961). Strain SL870 is an auxotrophic derivative of Iino's strain SW1061, which is a spontaneous *ah1*$^-$ mutant of LT2; we shall indicate its mutant allele as *ah1-1*. SW547 is a naturally occurring *ah1*$^-$ strain, not derived from strain LT2 (Iino, 1961); we shall indicate its mutant allele as *ah1-3*.

The 'phase-1-curly' mutant used was SW577, a *Salmonella typhimurium* strain (not derived from LT2) shown by Iino (1962) to be normally motile and with normal flagella when in phase 2, but almost non-motile and with short-wavelength flagella when in phase 1. As its abnormal character probably results from mutation in the structural gene for phase-1 flagellin, we shall indicate its genotype as *H1-i(curly)*.

The strains in which the original antigens of *Salmonella typhimurium* had been

replaced by other antigens, derived from strains of other species, had been obtained by transduction using phage P22, but were non-lysogenic for this phage. The source of the introduced *H1* and *H2* alleles, *H1-b* and *H2-e,n,x*, was *S. abony* strain SW803 (Spicer & Datta, 1959). *H1-a* came from *S. abortus-equi* strain SL23. This strain, like other strains of this species, is stable in phase 2. Furthermore, it carries an (unexpressed) phase-1-curly allele (Stocker, unpublished; Iino, 1962). However, the transductional clone we used though it had the phase-1 antigen *a* which is latent in the donor strain, SL23, did not have its (latent) phase-1-curly character—presumably because the *H1-a(curly)* allele of the donor had recombined with the *H1-i* allele of the LT2 recipient to produce an *H1-a* (non-curly) gene. The uncommon *H1* allele *H1-1,2* came from a monophasic-phase-1 strain of *S. paratyphi* B, strain CDC-15. This allele determines the production of phase-1 flagella which in antigenic character (Lederberg & Edwards, 1953) and amino-acid composition (McDonough, 1965) are indistinguishable from phase-2 flagella of antigenic type *1,2*, determined by the common *H2* allele *H2-1,2*.

Transduction. Phage P22 was used for transduction. The phage was propagated by the soft-agar-layer method on the strain to be used as donor (in some experiments re-isolated from a single colony in phase 1 or in phase 2). Lysates were sterilized by filtration or heat treatment. Recipient strains were used as broth cultures, inoculated from single colonies in the desired antigenic phase—or latent phase, in the case of non-flagellate recipient strains. To effect transduction, phage was added at a multiplicity of 5–10, and the mixture held at 37° for 10 min. Samples of serial decimal dilutions (usually three standard loopful amounts, each about 0·005 ml., on each of two plates for each dilution) were then inoculated on the surface of chilled semi-solid medium (Edwards & Bruner, 1942) in 5 cm. diameter plates. This medium is semi-solid at 37° but because of its high gelatin content it is solid at room temperature. The anti-H sera used, obtained from the Serological Standards Laboratory, Central Public Health Laboratory, Colindale, London, N.W. 9, had been so far as necessary cross-absorbed, and had agglutinating titres of 5000–20,000. The final concentration of a serum in the semi-solid medium used to test for inhibition of trail formation was between $\times 5$ and $\times 10$ its agglutinating titre. Testing of the flagellar antigens of swarms (complete *fla*$^+$, etc., transductants) confirmed that the sera at the concentrations used were effective and specific. The presence of trails and swarms was determined after overnight incubation at 37°, and the number of trails per arbitrary volume (6 loopful amounts) of undiluted transduction mixture was calculated from the number of trails seen on plates with few or no swarms. As the number of trails counted was necessarily very small, the estimate of trail frequency was of low precision.

In the genotype descriptions of abortive transductants the genes derived from the donor, present in the exogenote, are indicated by a subscript 1, and those of the recipient by a subscript 0.

RESULTS

Regulation of expression of exogenote H1 by the chromosomal phase-determinant

To test whether the phase-determinant, at or closely linked to *H2*, could regulate the expression of an *H1* allele in an exogenote fragment, we constructed abortive transductants, of constitution $fla_1^+ H1_1/fla^-_0 H1_0$, from an *H1*-linked *fla* recipient.

Strain *H1-iM12 fla-58* (SL831) was chosen as recipient, because it produces long trails and because *fla-58* is closely linked to *H1* (rate of cotransduction of *H1* with *fla-58* about 16%), so that nearly all transduced chromosomal fragments carrying *fla-58*$^+$ carry also the *H1* allele of the donor (Pearce & Stocker, 1965). Broth cultures grown from discrete colonies, of predetermined (latent) antigenic phase, were treated with phage P22 grown on phase-1 and phase-2 cultures of *fla*$^+$ donor strains having *H1-a* or *H1-b* as their *H1* allele, and with the *H2* allele *H2-e,n,x*. The ability of the transduction mixtures to produce trails in the presence of various sera and combinations of sera was then tested. Table 1 records the results with the donor strain carrying

Table 1. *Salmonella typhimurium. Effect of antisera on trails evoked from an H1-linked fla*$^-$ *recipient, in (latent) phase 1 or phase 2, by P22 lysates of phase-1 and phase-2 cultures of a fla*$^+$ *donor with a different phase-1 antigen*

Donor was LT2 *fla*$^+$ *H1-a H2-e,n,x* (= SL861). Recipient was LT2 *fla-58 H1-iM12 H2-1,2* (= SL831). Multiplicity about 10. Swarms were about one-tenth as abundant as trails; their expressed antigens indicated that the antisera, at the concentrations used, were active and specific. Trails from phase-1 recipient about 4 mm. long, those from phase-2 recipient about 6 mm. long (slightly shorter in the presence of anti-*a* serum, presumably because of a slight non-specific activity of this serum).

	Phase and relevant antigen of		No. of trails* in semi-solid medium with Serum for antigen determined by				
Line	Donor	Recipient	No serum	Recip. *H2* (anti-*1,2*)	Recip. *H1* (anti-*i*)	Donor *H1* (anti-*a*)	Donor and recip. *H1* (anti-*a* and anti-*i*)
a	Ph. 1(*a*)	Ph. 2(*1,2*)	3000	50	3000	4000	4000
b	Ph. 2(*e,n,x*)	Ph. 2(*1,2*)	3000	30	3000	3000	2000
c	Ph. 1(*a*)	Ph. 1(*i-M12*)	3000	3000	1	9	0
d	Ph. 2(*e,n,x*)	Ph. 1(*i-M12*)	8000	9000	0	8	0

* Calculated no./six loopfuls (about 0·003 ml.) of undiluted transduction mixture.

H1-a; the results with the *H1-b* donor were similar. In the absence of any antiserum the number of trails was about the same for all combinations of phase of donor and recipient. The susceptibility of the trails to antisera varied according to the phase of the recipient culture, but was not affected by the phase of the donor. Thus, when the recipient was in (latent) phase 2 (Table 1, lines *a* and *b*), the number of trails was not obviously reduced by the presence of antiserum for the phase-1 antigen of the recipient, or of antiserum for the phase-1 antigen of the donor, or of antisera for both phase-1 antigens—but was reduced about 99% by anti-*1,2* serum, active on the phase-2 antigen of the recipient. This result indicates that nearly all *fla-58*$^+$ abortive transductants arising from recipient cells in phase 2 express their chromosomal *H2* allele; but express neither their chromosomal *H1* allele nor the donor *H1* allele which nearly always accompanies *fla-58*$^+$ in the exogenote fragment. The few trails not inhibited by antiserum for the recipient phase-2 allele probably arose from a minority of recipient cells in phase 1; their proportion (about 1%) corresponds approximately to the proportion of phase-1 cells predicted by the rate of change from phase 2 to phase 1 in *Salmonella typhimurium* (Stocker, 1949).

When the recipient culture was in (latent) phase 1, the presence of antiserum for the

recipient phase-2 antigen did not decrease the number of trails (Table 1, lines *c* and *d*); thus, as expected, most or all *fla-58*$^+$ abortive transductants arising from phase-1 cells did not express their *H2* allele. The presence of antiserum for the donor phase-1 antigen decreased the number of trails by about 99·7 %, while the presence of antiserum for the recipient phase-1 antigen almost entirely prevented the appearance of trails. This indicated that nearly all *fla-58*$^+$ abortive transductants arising from phase-1 cells expressed both their chromosomal *H1* allele and the donor *H1* allele which accompanies *fla-58*$^+$ in the exogenote fragment. The few (about 0·3 %) trails unaffected by donor phase-1 antiserum probably resulted from a small proportion of transduced chromosome fragments which contained *fla-58*$^+$ but not *H1* (Pearce & Stocker, 1965). The single trail not suppressed by antiserum for the recipient phase-1 allele probably arose from a recipient cell in phase 2. The lack of effect of the phase of the donor on the phenotype of *fla*$^+$ abortive transductants is expected if phase is determined only at (or close to) *H2*, since no chromosomal fragment carrying *fla-58*$^+$ would carry also the unlinked *H2* region of the donor. It therefore appears that in *fla*$^+$ abortive transductants carrying two *H1* alleles they are both expressed if the cell is in phase 1, but neither is expressed if it is in phase 2.

Effect of ah1$^-$*, in cis and in trans, on the expression of H1*

When phase-1 (and therefore non-motile) cultures of *ah1*$^-$ recipients are treated with lysates of *ah1*$^+$ donors, abortive *ah1*$^+$ transductants are detectable because they produce trails We investigated the effect of antisera on such trails, to see whether an exogenote *ah1*$^+$ allele would permit expression of an *H1* allele adjacent to *ah1*$^-$ in the chromosome. We used as recipients two *Salmonella typhimurium* strains, with the *ah1*$^-$ alleles which we designate *ah1-1* and *ah1-3* (Iino, 1961); in both strains the unexpressed phase-1 antigen is *i*. The *ah1*$^+$ strains used as donors all had the same *H2* allele as the recipients, *H2-1,2*. One donor strain, LT2, had the same phase-1 antigen determinant, *H1-i*, as the recipients; the other, UP158, carried *H1-a*. The recipient cultures were in phase 1 and therefore non-motile. Anti-*1,2*-serum, active on the phase-2 antigens of all the strains, was included in the semi-solid medium, to prevent spreading of any cells changing into phase 2, by spontaneous change of phase or by transduction of an 'active' *H2* locus. When the donor carried *H1-i*, determining the same phase-1 antigen as the unexpressed phase-1 antigen of the *ah1*$^-$ recipients, the presence of anti-*i*-serum decreased the number of trails from thousands to zero (Table 2, lines *a* and *c*). When the donor carried instead *H1-a*, the presence of anti-*i* serum did not detectably decrease the number of trails, but anti-*a* serum prevented the appearance of any trails (Table 1, lines *b* and *d*). In a similar experiment, strain SD7, *H1-b*, was used as donor, and anti-*b* serum in place of anti-*a*; the results were qualitatively similar. It thus appears that *ah1*$^+$ abortive transductants express the *H1* allele adjacent (*cis*) to *ah1*$^+$ in the exogenote fragment; but that the *H1* allele adjacent to *ah1*$^-$ in their chromosome remains unexpressed, despite the presence of *ah1*$^+$ in the exogenote, i.e. in *trans*.

A corresponding result was found when *ah1*$^+$ was in the chromosome and *ah1*$^-$ in the exogenote. The strain used as recipient was non-motile because of the *H1*-linked *fla-66*, and carried *H1-b* (and *ah1*$^+$). Cultures in (latent) phase 1 were treated with P22 lysates of the two *ah1*$^-$ strains, and, as a control, of an *ah1*$^+$ strain, all having the *H1* allele *H1-i*. Serum for the (latent) phase-2 antigen of the recipient strain was

included in all plates, to prevent production of trails or swarms by any *fla*$^+$ transductants arising from recipient cells passing into phase 2. Nearly all transduced fragments carrying *fla-66*$^+$ carry also *H1* (Pearce & Stocker, 1965) and, presumably, *ah1*, which is very closely linked to *H1*. Whatever the donor strain, the presence of antiserum for the recipient phase-1 antigen prevented all trail formation (Table 2, lines *e–g*), i.e. as expected, all the *fla*$^+$ abortive transductants expressed the recipient *H1* allele, adjacent to its *ah1*$^+$. When the donor was *ah1*$^+$, anti-*i* serum (corresponding to the donor *H1* allele) decreased the number of trails by about 99% (Table 2, line *e*); thus nearly all *fla*$^+$ abortive transductants exressed the *H1* allele adjacent to *ah1*$^+$ in the *fla*$^+$ exogenote fragment. By contrast, when the donor was *ah1-1* or *ah1-3* (Table 2, lines *f* and *g*), *anti-i* serum did not detectably decrease the number of trails, nor make them shorter. Thus in all or most of the *fla*$^+$ abortive transductants the *H1-i* allele adjacent to *ah1*$^-$ in the exogenote remained unexpressed, despite the presence of *ah1*$^+$ in the chromosome. It appears, then, that an *H1* allele, in the chromosome or exogenote, cannot be expressed unless it is adjacent (*cis*) to *ah1*$^+$.

Table 2. *Salmonella typhimurium. Effect of antisera on trails arising (i) from* $ah1_1^+$ $H1_1/ah1_0^-$ $H1_0$ *abortive transductants; (ii) from* fla_1^+ $ah1_1^-$ $H1_1/fla_0^-$ $ah1_0^+$ $H1_0$ *abortive transductants*

The *ah1*$^-$ recipient cultures were phase 1, and therefore non-flagellate. The *fla*$^-$ recipient culture was in (latent) phase 1. Trails derived from SL870 (*ah1-1*) were about 3 mm. long, those from SW547 (*ah1-3*) about 5 mm. long, and those from SL696 (*fla-66*) about 7 mm. long.

Strain (genotype and strain no.)		No. of trails* in semi-solid medium with serum for recipient phase-2 antigen and:			
			Antiserum		
Recipient	Donor	No phase-1 antiserum	for recipient phase-1	for donor phase-1	Line
(i) $ah1_1^+/ah1_0^-$ trails					
ah1-1 H1-i (SL870)	*ah1*$^+$ *H1-i* (LT2)	7000	0	0	*a*
	ah1$^+$ *H1-a* (UP158)	500	500	0	*b*
ah1-3 H1-i (SW547)	*ah1*$^+$ *H1-i* (LT2)	1300	0	0	*c*
	ah1$^+$ *H1-a* (UP158)	140	160	0	*d*
(i) fla_1^+/fla_0^- trails					
fla-66 ah1$^+$ *H1-b* (SL696)	*fla*$^+$ *ah1*$^+$ *H1-i* (LT2)	4000	0	40	*e*
	fla$^+$ *ah1-1 H1i-* (SL870)	3000	0	6000	*f*
	fla$^+$ *ah1-3 H1-i* (SW547)	3000	0	3000	*g*

* Calculated as in Table 1.

Control of H2 by the phase-determinant in H2 abortive transductants

As the locus determining the phase of a bacterium is located either at *H2* or very close to it (Lederberg & Iino, 1956) an abortive *H2* transductant would contain two phase-determinants, one at or near *H2* in the chromosome, the other at or near *H2* in the exogenote. If the regulation of expression of *H2* by the phase-determinant is mediated by a cytoplasmic regulatory substance then in an *H2* abortive transductant either both or neither *H2* allele would be expressed. But if the phase-determinant

regulates directly the expression of the *H2* gene which it adjoins (or of which it forms part) the two *H2* alleles in such a cell would be independently regulated, and one might be expressed while the other was unexpressed. Determination of the flagellar antigens of *H2* abortive transductants should therefore show whether the regulation of *H2* is direct or indirect. A recipient non-motile culture which became motile on transduction of *H2* from a suitable donor would be expected to give also trails, in consequence of abortive transduction of *H2*. As none of the known *fla* or *mot* loci are co-transducible with *H2*, it was necessary to use a strain non-motile through some other cause. In one experiment we used as recipient a phase-1 culture of strain SL870, of genotype *ah1*$^-$ *H1-i H2-1,2* and therefore non-motile when in phase 1. This culture was treated with P22 lysates of phase-1, and of phase-2, cultures of SL861, a motile LT2 line whose H alleles are *H1-a* and *H2-e,n,x*. All the plates of semi-solid medium contained sufficient anti-*1,2* serum to prevent the spread of any recipient bacteria which became motile by spontaneously passing into phase 2. On this medium the recipient bacteria treated with the phase-1 lysate produced many trails (calculated number from a standard inoculum, about 8000; Table 3, line *a*). The additional presence in the semi-

Table 3. *Salmonella typhimurium. Effect of antisera on trails evoked from phase 1 (and therefore non-motile) ah1*$^-$ *and H1-curly recipients by P22 lysates of phase-1 and phase-2 cultures of donor with different phase-1 and phase-2 antigens*

Donor was LT2 *H1-a H2-e,n,x* (SL861). The trails produced by SL870 were about 3 mm. long; those produced by SW577 about 4 mm. long.

Recipient (genotype and strain no.)	Donor, phase and relevant antigen	No. of trails* in presence of serum against H antigen(s) determined by recip. *H2* and: No further serum	Recip. *H1* (anti-*i*)	Recip. *H1* and donor *H1* (anti-*i* + anti-*a*)	Donor *H2* (anti-*e,n,x*)	Donor *H2* and donor *H1* (anti-*e,n,x* + anti-*a*)	Line
H1-i ah1-1 H2-1,2 (SL870)	Ph. 1 (*a*)	8000	8000	12	8000	0	*a*
	Ph. 2 (*e,n,x*)	2000	3000	600	2000	0	*b*
H1-i-curly H2-1,2 (SW577)	Ph. 1 (*a*)	0	0	2	0	0	*c*
	Ph. 2 (*e,n,x*)	700	800	800	0	0	*d*

* Calculated as in Table 1.

solid medium of serum against the phase-2 antigen of the donor, or of antiserum for the unexpressed phase-1 antigen of the recipient, did not sensibly diminish the number of trails. The presence of anti-*a* serum, specific for the phase-1 antigen of the donor, decreased the number of trails from about 8000 to 12. We infer that nearly all the trails evoked by the phase-1 lysate arose from abortive transductants with no flagellar antigen other than that determined by the *H1* allele of the donor—the expected phenotype for $H1_1\ ah1^+/H1_0\ ah1^-$ abortive transductants in phase 1. In this experiment since both donor and recipient were in phase 1, all (or nearly all) *H2* abortive transductants would contain two 'inactive' *H2* alleles (and an *H1* allele unexpressed because of *ah1*$^-$) and would therefore remain non-flagellate and not produce trails. However, a very few trails (12 amongst 8000) were not inhibited by antiserum for the donor phase-1 antigen, and these few trails were inhibited when antiserum for the

donor phase-2 antigen was also present. It appears that though the donor was in phase 1 at the time of lysis, a small proportion (12/8000) of the *H2* abortive transductants expressed the donor phase-2 antigen. This proportion corresponds approximately with the proportion of phase-2 cells calculated to have been present in the phase-1 donor culture at the time it was infected with phage P22.

A different result was obtained when the phase-2 donor lysate was used (Table 3, line *b*). The presence of antiserum against the donor phase-1 antigen decreased the number of trails, but only by about 70% (from about 2000 to about 600), instead of by more than 99%. We infer that only about 70% of the trails evoked by the phase-2 lysate arose from *ah1*$^+$ *H1* abortive transductants. When antiserum for the donor phase-2 antigen was present, as well as antiserum for the donor phase-1 antigen, no trails were produced. We infer that the 30% of trail-forming cells which did not manifest the donor phase-1 antigen manifested instead the donor phase-2 antigen, in consequence of abortive transduction of *H2*. These trails were produced in the presence of antiserum for the recipient phase-2 antigen, added to prevent spread of recipient bacteria becoming motile by spontaneous passage into phase 2. The trail-producing cells concerned therefore showed the donor phase-2 antigen but not the recipient phase-2 antigen. Thus an exogenote *H2* transduced from a donor in phase 2 expressed itself in a recipient cell which was in phase 1, despite the presence of its 'inactive' chromosomal *H2*, which remains unexpressed.

Control of the expression of endogenote H1 by exogenote H2

The phase-determinant at or near *H2* controls the expression of *H1* via the cytoplasm (or more precisely not through the chromosome) presumably by controlling the production of a regulatory substance. Such a regulatory substance might be either a repressor of the activity of *H1*, produced only in cells in phase 2, or a positive regulator (internal inducer) of *H1*, produced only in cells in phase 1. In an abortive *H2* transductant having one phase-determinant in the phase-1 state and the other in the phase-2 state, the chromosomal *H1* gene would be unexpressed if the regulatory substance were a repressor—because the phase-determinant which was in the phase-2 state would determine production of the *H1*-repressor substance. If, on the other hand, the regulatory substance were an 'internal inducer', the chromosomal *H1* gene of such a cell would be induced by the inducer substance determined by the phase-determinant which was in the phase-1 state. The experiments just described (Table 3, lines *a* and *b*) do not distinguish these hypotheses because the *ah1*$^-$ character of the recipient strain would in any event prevent expression of its *H1* allele. We therefore wished to make similar experiments with an *ah1*$^+$ recipient; but it was necessary that this recipient, though in phase 1 and able to express its phase-1 antigen, should not spread through the semi-solid medium, even in the absence of homologous antiserum. We achieved this by use as recipient of a strain with the 'phase-1-curly' character. Such strains when in phase 2 produce normal flagella and spread normally, but when in phase 1 they make flagella with an abnormally short wavelength, show very little translational motility and spread very slowly or not at all in semisolid medium (Iino, 1962). Both genetical evidence (Iino, 1962; Joys & Stocker, 1963) and chemical evidence (Enomoto & Iino, 1966; Asakura, Eguchi & Iino, 1966) indicate that the phase-1-curly (or phase-2-curly) character arises from mutation within the structural gene for the corresponding phase-1 (or phase-2) flagellin, causing a change in the amino acid sequence of this

protein. Theoretical considerations, and observations on the trail-forming ability of *fla*$^{+}_{1}$ *H1-curly*$_{1}$/*fla*$^{-}_{0}$ *H1-curly*$^{+}_{0}$ abortive transductants, described below, indicate that abortive transductants synthesizing both an abnormal ('curly') flagellin and a normal flagellin make flagella containing both proteins, and that such bacteria show translational motility and produce trails. These, however, are abnormally short, presumably because flagella made from the mixture of normal and abnormal protein are less efficient locomotor organs than those containing only normal flagellin. Hence, the abortive transduction of an 'active' *H2* allele to a phase-1 (and therefore non-motile) culture of a phase-1-curly recipient would be expected to confer motility, and so to cause the appearance of trails inhibitable by antiserum for the donor phase-2 antigen; but if in such abortive transductants the chromosomal *H1-curly* allele was also expressed the trails would be abnormally short, and inhibitable also by antiserum for the recipient phase-1 antigen. We used these two criteria to test for expression of the chromosomal *H1-curly* gene in such abortive *H2* transductants, i.e. when the phase-determinant of the recipient was in the phase-1 state and that of the donor in the phase-2 state. The phase-1-curly recipient was SW 577. A phase-1 culture was treated with phase-1 and phase-2 lysates of an LT 2 line with the H alleles *H1-a* and *H2-e,n,x*. The semi-solid medium contained anti-*1,2* serum, specific for the phase-2 antigen of the recipient, to prevent the spreading growth of any recipient bacteria which might change into phase 2. When the phase-1 lysate was applied, only 2 trails were detected, both of them on medium containing antisera for the donor and for the recipient phase-1 antigens (Table 3, line *c*). The phase-2 lysate evoked numerous trails (Table 3, line *d*), of average length about 4 mm., i.e. not abnormally short as they would have been if the recipient *H1* allele had continued to direct the synthesis of an abnormal flagellin. Neither the number nor the length of the trails was decreased by the presence of antiserum for the phase-1 antigen of the recipient—nor by antiserum for the donor phase-1 antigen. Anti-*e,n,x* serum, corresponding to the donor phase-2 antigen, decreased the number of trails to zero. We infer that all the trails evoked by the phase-2 lysate arose from *H2* abortive transductants, all of them expressing the donor *H2* allele and none of them expressing either the *H1-curly* or the *H2* allele of the recipient. The exogenote *H2* derived from a donor in phase 2 thus expressed itself after introduction into a phase-1 recipient, and prevented the further expression of the previously expressed *H1* allele (in this case, the *H1-curly* allele) of the phase-1 recipient; but the chromosomal *H2* allele remained unexpressed. This shows that the phase-determinant regulates the expression of *H1* by determining the production of a cytoplasmic repressor of *H1*, not by controlling the production of a positive regulator (inducer) of *H1*, and confirms the inference that the phase-determinant regulates directly the expression of the *H2* gene which it adjoins or forms part of and does not regulate that of another *H2* allele present in the same cell.

In the experiments just described (Table 3, lines *c* and *d*), no trails expressing a phase-1 antigen or antigens, i.e. no abortive *H1* transductants, were detected, even though in the similar experiment with the *ah1*$^{-}$ recipient (Table 3, lines *a* and *b*) most of the trails were of this sort. The absence of *H1* abortive transductants is probably only apparent and results from the *H1-curly* character of the recipient. Phase-1 cultures of this strain, SW 577, are not entirely non-motile, and they spread very slowly in semi-solid medium. This slow spread does not interfere with the detection of trails of normal length (about 5 mm.). But in experiments with recipients non-motile

because of *fla*⁻ mutation (*fla-58* or *fla-66*) closely linked to *H1*, the *fla*⁺ abortive transductants evoked by an *H1-curly* lysate made trails which were very much shorter than those evoked by an *H1-curly*⁺ lysate, presumably because they are of constitution *H1-curly*$_1$ *fla*⁺$_1$/*H1-curly*⁺$_0$ *fla*⁻$_0$ and therefore make flagella containing both abnormal and normal flagellin, and are consequently less motile than bacteria whose flagella contain only normal flagellin. In the experiment in which the recipient was the phase-1-curly strain (Table 3, lines *c* and *d*) *H1* abortive transductants would likewise presumably have produced only short trails, and these might well have been obscured by the narrow halo of spread of the recipient strain.

Consideration of phase-2 flagellin as a possible repressor of H1

Bacteria which are in phase 2 and are flagellate produce both phase-2 flagellin and, we have inferred, a repressor of the expression of *H1*. A simple explanation would be that some of the phase-2 flagellin synthesized reaches *H1* via the cytoplasm and acts as the repressor of this gene. We tested this hypothesis by use of an exceptional strain of *Salmonella paratyphi* B, SW546 = CDC157, which has an *H1* allele determining flagellin of antigenic character *1,2* (Lederberg & Edwards, 1953), though antigen *1,2* in other strains of *S. paratyphi* B, *S. typhimurium* and many other *Salmonella* species is a phase-2 antigen, determined at the *H2* locus. The *1,2* flagellin determined by the exceptional *H1* allele cannot be distinguished from ordinary *1,2* flagellin, determined by *H2-1,2*, either serologically (Lederberg & Edwards, 1953) or in amino-acid composition (McDonough, 1965), and it seems likely that the flagellins determined by *H1-1,2* and by *H2-1,2* are identical. If the repressor of *H1* in ordinary diphasic strains of *S. paratyphi* B, etc., is their phase-2 flagellin *1,2*, then the supposedly identical *1,2* flagellin determined by *H1-1,2* should likewise repress the expression of a normal *H1* allele. To test this prediction, we made phase-1 abortive transductants of constitution *H1-1,2*$_1$ *fla*⁺$_1$/*H1-b*$_0$ *fla*⁻$_0$ and determined their flagellar antigen phenotype. The donor strain was an LT2 line given the *H1-1,2* allele by transduction, and with the *H2* allele *H2-e,n,x*. The recipient was a phase-1 culture of an LT2 line of genotype *H1-b fla-66 H2-e,n,x*. and nearly all phase-1 *fla*⁺$_1$/*fla-66*$_0$ abortive transductants express both the donor and the recipient phase-1 antigens. In the experiment with the *H1-1,2* donor, more than 99% of the trails were inhibited by anti-*1,2* serum, and more than 99% of them were also inhibited by anti-*b* serum. That is, the *fla*⁺ abortive transductants expressed both their phase-1 antigens and the *1,2* flagellin determined by the exogenote *H1-1,2* did not repress the expression of the normal *H1-b* allele in the chromosome. It is therefore unlikely that in ordinary diphasic strains the *1,2* (or other phase-2) flagellin is the repressor substance for *H1*.

DISCUSSION

Consider first the expression of *H1* as influenced by *ah1* and by *H2*. In *ah1*⁺ abortive transductants obtained from a phase-1 *ah1*⁻ culture, the *H1* allele adjacent to *ah1*⁻ in the recipient was unexpressed, though the *H1* allele adjacent to *ah1*⁺ in the exogenote was expressed (Table 2, lines *a–d*). Similarly in phase-1 *fla*⁺ abortive transductants the *H1* allele accompanying *fla*⁺ in the exogenote was unexpressed if it was adjacent to *ah1*⁻, but expressed if adjacent to *ah1*⁺ (Table 2, lines *e–g*). The non-expression of an *H1* allele adjacent (*cis*) to *ah1*⁻ despite the presence of an *ah1*⁺

allele in the same cell shows that *ah1* does not affect the expression of *H1* by means of a cytoplasmic regulatory substance, nor by coding for a product, such as a specialized ribosome, needed for expression of *H1* though not of *H2*. Nonsense or frame-shift mutations within the structural gene for phase-1 flagellin would account for the *ah1*$^-$ phenotype. However, *ah1* seems to be distinct from *H1* in that *ah1*$^-$ mutants retain the genetic information for making the flagellar antigen determined by their *H1* allele, as is revealed by the phase-1 flagellar antigen of their *ah1*$^+$ revertants and of recombinants in which there has been crossing-over between the determinant of phase-1 antigenic specificity and *ah1* (Pearce & Stocker, unpublished observations). The site of *ah1* mutation may be an *H1* 'operator' locus, comprising DNA whose function is to recognize and react with the *H1* repressor substance. The normal phase variation of the exceptional strains which have antigen *1,2* as a phase-1, instead of as a phase-2, antigen suggests that they have a normal *H1* operator, which does not form part of the flagellin structural gene. However, it is possible that the repressor recognition site, assumed to be altered in *ah1*$^-$ mutants, is within the flagellin structural gene, but in a part of it which codes for an antigenically null region of the polypeptide chain, perhaps at or near its N terminus.

The experiments on the flagellar antigens of abortive transductants from an *H1*-linked *fla*$^-$ recipient (Table 1) tested the ability of the phase-determinant of the recipient to regulate a non-chromosomal *H1* allele. Antiserum for the donor phase-1 antigen inhibited trails evoked by lysates of a donor in phase 1 or phase 2 when the recipient was in latent phase 1, but not when it was in latent phase 2. Thus expression of the exogenote *H1* was controlled by the phase of the recipient, i.e. by the 'state' of its phase-determinant at *H2*, just as was that of the chromosomal *H1* allele. The non-expression of the chromosomal *H1* allele in *H2* abortive transductants evoked from a phase-1-curly recipient strain in phase 1 by a lysate of a donor in phase 2 (Table 3, line *d*) shows that the expression of a previously active chromosomal *H1* allele is prevented by an exogenote active *H2* allele. These observations show that the control of expression of *H1* by the state of *H2* (otherwise stated, the epistasis of *H2* to *H1*) does not depend on their locations on the chromosome, causing, for instance, more *H2* than *H1* messenger RNA to be made (Iino & Lederberg, 1964). They can be explained by several sorts of cytoplasmic interaction. For instance, *H2* messenger RNA, made only by cells with an active *H2* allele, might compete successfully with *H1* messenger RNA for attachment to a specialized ribosome used in synthesis of flagellin (Iino & Lederberg, 1964). However, analogy with other systems makes it plausible to suppose that the expression of *H1* is determined by an *H1* regulatory substance, whose production or non-production is determined at, or close to, *H2*. This regulatory substance might be either a positive regulator ('internal inducer') such as is involved in the regulation of the arabinose genes in *E. coli* (Englesberg *et al.* 1965)—or a repressor, such as that which regulates the lactose genes in the same organism. Either kind of regulatory mechanism would account for the control of exogenote *H1* by endogenote *H2*. However, the non-expression of the previously active *H1* allele in the *H2* abortive transductants evoked from a phase-1 culture of a phase-1-curly recipient indicates that the regulation of *H1* is effected by a repressor substance, produced by active *H2* regions—for if it was effected by a positive regulatory substance the inactive (since unexpressed) *H2* in the chromosome of the abortive transductants would presumably have continued to determine production of the

'inducer' of *H1*, which would therefore have been expressed. It therefore appears that *H2* does not control *H1* by controlling production of a positive regulatory substance but, probably, by controlling production of a repressor of expression of *H1*.

The expression of the donor phase-1 antigen when an *H1* allele from a phase-2 donor was present in the exogenote in a *fla*$^-$ recipient in phase 1, and the non-expression of an *H1* exogenote allele derived from a phase-1 donor in a phase-2 cell of the same recipient strain indicates that regulation of expression of an *H1* allele can be effected by the phase-determinant in the recipient cell even though the *H1* allele concerned is never replicated. This parallels other examples, where environmentally determined repression or de-repression of a gene or operon is not dependent on its replication.

Consider now expression of *H2*. The *H2* abortive transductants evoked from phase-1 cultures of *ah1*$^-$ and phase-1-curly recipients by treatment with phase-2 lysates expressed the donor *H2* allele present in the exogenote, but the recipient *H2* allele in the chromosome remained unexpressed. Thus, the expression of an *H2* gene depends on its inherent state (or on the state of its adjacent (*cis*) phase-determinant locus) and is unaffected by the state of another *H2* region in the same cell. This is incompatible with the suggestion (Klein, 1964) that the non-expression of *H2* in phase-1 cells results from the presence in them of a cytoplasmic repressor of *H2*. It suggests, on the contrary, that there is an *H2* operon with two metastable states, the active or phase-2 state corresponding to wild-type and the inactive or phase-1 state to that of an operator-negative mutant.

We have argued above that *H2*—or a phase-determinant locus closely linked to it—regulates expression of *H1* by determining production or non-production of an *H1*-repressor substance. The simplest hypothesis, that the *H1*-repressor substance is *H2* messenger RNA or *H2* flagellin, is not supported by our observation that an ordinary *H1* allele in the chromosome and an exceptional *H1* allele, determining what is usually a phase-2 flagellin, are both expressed in *fla*$^+$ abortive transductants. If the messenger RNA and *1,2* flagellin products of the uncommon *H1-1,2* allele are identical with those of the common *H2-1,2* allele then the chromosomal *H1* allele, *H1-b*, would have been repressed if *H2* messenger RNA or *H2* flagellin is the repressor of *H1*. However, the evidence for the identity of flagellin *1,2* determined by *H1-1,2* with that determined by *H2-1,2* does not exclude the possibility of a minor difference in amino-acid sequence, at one end, for instance, which might be antigenically null but affect repressor activity. However, we consider it more likely that there is a special repressor substance, probably a protein, coded for at an *H1*-repressor locus closely linked to *H2* and forming part of the same operon as it. It is then necessary to postulate that the activity or inactivity of this *H2* operon depends on the state of a metastable operator region, which alternates between an 'on' or 'active' configuration, corresponding to wild-type, and an 'off' or 'inactive' configuration, corresponding to that of an operator-negative mutant. It seems unlikely that the postulated metastable region is comprised within the structural gene for phase-2 flagellin, because the exceptional *H1* allele *H1-1,2*, which presumably arose by translocation of an ordinary *H2-1,2* gene, is not metastable. A gene which alternates in a random or mutation-like manner between two alternative states has been reported in *Neurospora crassa* (Barnett & de Serres, 1963). As in the case of the *H2* operator it is not known whether the change from one state to the other depends on a base-substitution or on some local rearrangement, for

instance, an inversion. So far as we know, an operon or unit of expression comprising both a structural gene and a repressor for an unlinked gene has not previously been reported. Such an element might well be involved in the complex regulatory processes of differentiation in higher organisms. The phenomenon of perithecial-aperithecial variation in *Aspergillus nidulans* may depend on the intermittent expression of a gene which determines some cytoplasmic factor affecting perithecial development (Croft, 1967).

The above interpretation of phase-variation requires some modification of the proposals of Iino & Lederberg (1964) on the evolution of this phenomenon. The existence of numerous *Salmonella* species without an *H2* locus, and the homology of the single flagellar antigen locus *H* of the monophasic species *Escherichia coli* with *H1* of Salmonella (Mäkelä, 1964) suggest that *H2* arose by a duplication and translocation of a primitive *H1* gene. The response of the *H1* of monophasic Salmonella and of *H* of *E. coli* to the *H1*-repressor substance determined at or close to *H2* (Mäkelä, 1964) implies that the primitive *H1* locus, and so presumably also its duplication which became *H2*, had adjacent to it an operator which responded to an *H* regulatory substance. The non-production of flagella (of either phase) in certain environments, e.g. at 44° in *S. typhimurium* or on simple defined medium in *S. typhi*, and the apparent absence of flagellin in the cytoplasm of mutants lacking flagella because of mutation at any of several *fla* loci (Iino & Enomoto, 1966) suggest that there is a regulatory system governing expression of both *H1* and *H2*—probably involving a repressor substance active on both loci. No gene for production of such a repressor of both *H1* and *H2* has been discovered or mapped—but analogy with the *lac* cluster and the presence near *H1* of many genes concerned with various flagellar characters suggest that in a primitive monophasic species a regulatory gene might have been adjacent to *H1*, and therefore involved in the duplication and translocation which produced *H2*. The repressor-recognition site (operator?) of *H2*, and the postulated *H2*-linked duplication of the *H* regulatory gene *H1* might then have changed in such a way that the product of the *H2*-linked regulatory gene continued to repress *H1* but no longer repressed *H2*—thus accounting for the origin of the *H2*-linked *H1*-repressor locus. A local rearrangement at the *H2* region might then have brought this *H1*-repressor locus, previously merely linked to *H2*, into the same operon as it—the situation which we now postulate. If the ability to manifest alternately two different flagellar antigens were for unknown reasons advantageous, then mutations which altered the operator region of the *H2* operon in such a way that it spontaneously alternated between metastable 'on' and 'off' configurations would be preserved, and would account for phase-variation.

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