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The Development of Rumen Microbial Populations in Lambs and Calves Under Various Conditions of Management

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SUMMARY

The development of the rumen flora and fauna has been followed by microscopic observations of rumen fluid from 58 young lambs and calves kept under different conditions of management; including remaining with the dam, early weaning and artificial inoculation into the rumen. Ciliate-free animals were successfully maintained by isolation from direct contact with other ruminants from an early age, but it was noted that greater space should be left between cattle than between sheep. The general effect of different diets and treatments proved similar in calves and lambs but certain differences were noted in their flora. Certain large bacteria developed in isolated lambs without direct contact between animals. Some generalizations, particularly the effect of a high concentrate diet, were applicable to all animals examined, but there were variations between individual animals. In the establishment of rumen ciliates diet was the governing factor and artificial inoculation with ciliates proved as effective as constant contact with the dam. Although the ciliate-free animals showed no differences in performance certain differences in the rumen flora common to isolated calves and lambs were noted.

INTRODUCTION

The normal adult ruminant harbours an active mixed microbial population of bacteria and protozoa which play a significant role in the breakdown of the components of the ration. There is a range of morphologically different species of bacteria (see, for instance, Moir & Masson, 1952; Smiles & Dobson, 1956), and the principal protozoa are ciliates, although some small flagellates are also present. The rumen ciliates are of two families: the highly complex Ophryoscolecidae of which there are many species and the holotrich species *Isotricha prosoma*, *I. intestinalis* and *Dasytricha ruminantium* which belong to the Trichostomatidae. It has been said that this mixed microbial population is not found in the very young milk-fed animal but is first observed when the rumen itself has enlarged and solid food has been consumed.

Considerable interest has been shown in the development of rumen microbial populations in calves kept on different rations and under varying conditions of management (Pounden & Hibbs, 1948*a*, *b*, 1949, 1950; Bryant, Small, Bouma & Robinson, 1958; Bryant & Small, 1960). The value of artificial inoculations of rumen contents in early weaning has been studied, as for example by Preston (1958), but the low rumen pH proved to be a limiting factor in the establishment of an adult type of microbial population. Pounden & Hibbs (1948*a*, *b*) and Eadie,

Hobson & Mann (1959) have noted the significance of the ration in regulating the pH and hence the microbial population in calves and Purser & Moir (1959) have demonstrated the relationship between minimum rumen pH and ciliate numbers in mature sheep. Phillipson (1955) quotes the range of pH values within the rumen for animals on a normal ration as 5·9–7·4 for cattle and 5·3–7·3 for sheep, and Briggs, Hogan & Reid (1957) who obtained pH values as low as 4·5 for sheep on certain rations considered that a rumen pH below 5·0 was not normal for an adult animal.

The microbial populations of different domestic ruminant species have generally been considered to be strictly comparable yet no detailed comparison has been made and no one has studied the rumen ciliate populations *in vivo* from the point of view of inter-relationships between the component species.

This paper records a study of the development of the rumen microbial population in a total of 58 young ruminants—calves and lambs—kept under various conditions of management including the natural one of remaining with the dam. The questions considered to be of special interest in this study were to what extent the microbial population was governed by age of host, diet, availability of micro-organisms and species of ruminant and in addition whether there were individual variations between animals. Particular note has been taken firstly of the development of organisms under different treatments and secondly of any striking inter-relationships between bacteria and ciliates. In a second paper (Eadie, 1962), observed inter-relationships between the different species of ciliates within these young ruminants are discussed. Finally, general comparisons have been made with calves and lambs kept completely free from ciliates by isolation.

Becker & Hsiung in 1929 first demonstrated that the rumen ciliates are only passed from animal to animal by direct transfer between hosts, of saliva containing the active organisms. Resistant cysts have never been found and viable rumen ciliate forms are not present either in the food or faeces. The active organisms which reach the host's mouth during rumination are rapidly killed by drying or exposure to air. Thus transfer is normally effected by direct mouth-to-mouth contact between animals. This feature of the rumen ciliates has greatly facilitated the present study since it is possible to control the ciliate fauna by appropriate spatial separation of animals.

METHODS

Animals

Young animals

Table 1 lists the lambs and their management, Table 2 the early weaned calves and Table 3 the twin calves which were weaned at a later stage; one further calf, 'Z', was maintained free from ciliates on the late weaning system. All animals received colostrum for at least 3 days and thereafter, where milk was fed, whole cows' milk was used. The lambs from any 1 year were born within a short time of each other and were therefore studied over much the same period of time.

Adult rumen-cannulated sheep

Throughout the period of the experiments a number of adult rumen-cannulated sheep were maintained to provide rumen fluid from which the inocula were prepared. Some of these were fed on hay and a cereal-based diet and the remainder

on hay and grass cubes. Three partially-faunated sheep in which limited ciliate populations (see (*C*), (*D*) and (*E*) p. 566), had been established by the method of Eadie & Oxford (1957), were maintained in isolation.

Table 1. *Rations and management of lambs*

Year	Breed	Lamb no.	Management*	Age at time of first inoculation of rumen ciliates plus bacteria (days)	Age regular sampling started (days)	Age stomach tube samples terminated (days)
1956	Blackface	50	D	42	42	223
	Blackface	51	D	42	42	223
1958	Shetland × Cheviot	305	B Weaned 135 days	147	27	167
	Shetland × Cheviot	327	B Weaned 145 days	177	22	295
1959	Cheviot	90 T1	A	—	7	91
	Cheviot	92 T2	A	—	6	90
	Cheviot	94	A	—	9	100
	Cheviot	91 T1	C	8	6	141
	Cheviot	93 T2	C	7	5	133‡
	Cheviot	95	CE	—	8	134‡
	Cheviot	96	CE_1	678	7	133‡
	Cheviot	88	B Weaned 118 days	—	7	133
	Cheviot	89§	B Weaned 118 days	113	8	174
	Blackface	411†	B	—	7	88
	Blackface	418†	B	—	7	88
1960	Blackface	221	CE	—	22	205‡
	Blackface	222§	CE_2	182	22	233
	Border Leicester × Cheviot	230§	C	18	18	185‡
	Border Leicester × Cheviot	231§	C	18	18	211
	Border Leicester × Cheviot	232§	C	14	14	207‡
	Blackface	241 T3	B	—	9	44
	Blackface	242 T3	B	—	9	44
	Blackface	245 T4	B	—	10	42
	Blackface	246 T4	B	—	10	42
	Blackface	247	B	—	9	44

* A, with ewe, outdoors on grass; B, with ewe, indoors with access to ewe's concentrates; C, bottle-fed whole milk, grass *ad lib* at 21 days, weaned on to concentrated lamb ration + *ad lib* grass at 93 days; D, bottle-fed, weaned at 77 days on to hay *ad lib* + concentrate mixture; E, completely isolated; E_1, completely isolated until 678 days; E_2, completely isolated until 182 days.

† Ewe on low calcium and phosphorus ration and milk yield was rather small.

‡ Animal cannulated and kept on further experiments.

§ Used for antagonism experiments (Eadie, 1962).

} Creep fed together with concentrates, from 44 days.

T1, etc., Twins.

[In the same pen together.

The ciliate populations available for inocula were therefore as follows:

(*A*) Entodinium and holotrich species with *Polyplastron multivesiculatum* (Dogiel & Fedorowa) as the predominant large Ophryoscolecid with various other ciliates such as *Diploplastron affine* (Dogiel & Fedorowa) and, in early experiments, *Ophryoscolex tricoronatus* (Dogiel).

(*B*) Entodinium and holotrich species with the large Ophryoscolecid genera *Eudiplodinium* and *Epidinium* either together or separately.

(B_1) A population like *B* but without holotrich organisms.

(*C*) Entodinium species alone (one isolated animal).

(*D*) Entodinium species, *Dasytricha ruminantium* and Epidinium spp. (one isolated animal).

(*E*) Entodinium species, *Isotricha prostoma*, *I. intestinalis* and *Polyplastron multivesiculatum* (one isolated animal).

Table 2. *Calves—early weaning treatments*

The numbers under the treatment headings are those by which the calves are mentioned in the text.

(*a*) Grass available at 7 days. Concentrates + 0·8 g./lb. aureomycin at 15 days. Weaned at 21 days	(*b*) As (*a*) but without aureomycin	(*c*) Concentrates available at 7 days. Weaned on to all concentrates at 21 days	(*d*) Grass at 7 days Weaned on to varied ratios of grass: concentrates at 21 days
2	86	610	4*†
3*†	—	611	5
6	—	612	7
8	—	613	21
71	—	—	35†
78	—	—	72
79‡	—	—	84 T1
80	—	—	—
81‡	—	—	—
85 T1	—	—	—

* See Eadie, Mann & Hobson (1959).
† Used in antagonism experiments (Eadie, 1962).
‡ Fed milk by teat whereas all the others fed milk by bucket.
T, Twins.
N.B. All Ayrshire except 612, Friesian, and 613, Aberdeen Angus.

Housing

Normal

The early-weaned calves and the mature cannulated sheep were kept in separate pens with wooden sparred partitions so that there was some contact between neighbouring animals of the same age. This also applied to the lambs 411, 418, 241, 242, 245, 246 and 247 which were kept in pens with the ewes. The other lambs kept indoors with the ewe were fairly well isolated from contact with other animals as were the lambs kept together in the same pen. The lambs and ewes out on grass were in the same field as Shetland ewes but were never seen to mingle very much.

Isolation

The development of a routine procedure by which animals could be successfully

Table 3. *Late weaning treatments of identical twin calves*

Calf breed	Grass or hay first available at (days)	Concentrates first available at (days)	Weaned at (days)	Period of time kept unfaunated (days)	First sample at (days)	Inoculated (days)	Special treatment	Age when regular samples terminated (days)
A } Shorthorn	Hay 24	35	105	—	17	From 24	Fed by bottle	171
B } Shorthorn	Hay 24	35	105	—	17	From 24	Fed by bucket	171
X } Ayrshire	Hay 27	56	117	—	21	From 21	Faunated *v.* unfaunated. On indoor normal ration	228
Y } Ayrshire	Hay 27	56	117	To 84	21	On 84 and 214		228
P } Ayrshire	Hay 34	62	123	—	23	From 23	Faunated *v.* unfaunated. On indoor normal ration	207
Q } Ayrshire	Hay 34	62	123	To 98	23	On 98 and 193		207
N* } Jersey	Grass 35	35	99	—	14	From 42	Faunated *v.* unfaunated. Out on to grass suddenly	337
O* } Jersey	Grass 35	35	99	To 163	14	With other calves from 164		337
F* } Ayrshire × Friesian	Grass 39	41	95	—	8	From 42	Faunated *v.* unfaunated. On low energy hay ration from 169	438
G* } Ayrshire × Friesian	Grass 39	41	95	To 196	8	From 274		438

* Used in antagonism experiments (Eadie, 1962).

} Twin calves.

N.B. 'Unfaunated' in this Table means free from ciliate protozoa. All except A were fed milk by bucket. Concentrate ration was the same throughout and was also used for lambs (Table 1).

and indefinitely maintained completely free from rumen ciliate contamination was carried out over a long period of time and some modifications in management and design of housing proved necessary. The object was to prevent physical contact between animals at all times and to avoid chance transfer of live ciliates in saliva or rumen liquor during cleaning, feeding or sampling. At the same time in order to obtain otherwise healthy normal animals it was advisable to leave them within sight and sound of other animals and in addition for this work it was necessary not to eliminate the possibility of aerial transfer of rumen bacteria. Some authors (Bryant & Small, 1960) have suggested that it is essential to use disinfectants on workers and apparatus together with changing of clothes before animals are handled. In the present work constant general care in the order of handling the animals, and rigorous washing of all contaminated material with soap and water, has proved adequate.

(*a*) *Partially faunated isolated cannulated sheep.* These sheep were kept in the same room as other mature sheep but the most satisfactory housing proved to be a wooden crate similar to a metabolism crate. This was placed within a solid partitioned pen (see (*b*) below). The crates were sufficiently narrow so that the sheep could not turn and they were placed in line; nose to tail; so that each sheep was 5 ft. from the rear of the crate in front.

(*b*) *Isolated lambs.* From up to 3 days of age the ciliate-free lambs numbers 95, 96, 221 and 222 (Eadie & Hobson, 1962) were kept isolated in pairs in pens with high wooden partitions made from closely fitting, interchangeable, smooth boards. These pens were specially designed for this work. Since animals tended to chew through even this smooth wood some of the more accessible boards were covered with thin steel sheeting. As with the partially faunated sheep mature animals were present in the same room.

After rumen cannulation and when sufficiently large, lambs 95 and 96 were transferred to crates identical with those used for the sheep mentioned in (*a*).

(*c*) *Isolated calves.* Each member of a pair of twins was housed and managed in the same way except that extra care was taken to prevent contamination of the ciliate-free animals. The calves *P*, *Q*, *X* and *Y* were tethered in solid partitioned cow stalls but these were unsatisfactory when the animals grew since contact became possible and the animals were too accessible to visitors, or to chance stray ruminants. The pens used for *N*, *O*, *F* and *G* were of the design used for the young isolated lambs but were necessarily larger. A sealed perspex window was fitted into one of the boards of the partitions to allow the animals to see their neighbours. The calves were tethered in a position of nose to tail.

Sampling and inoculation

Samples were initially obtained by polythene stomach tube from all the young animals although some were later fitted with rumen cannulae. When using a stomach tube on young lambs it is possible to obtain abomasal—not ruminal samples but such samples could be recognized by the low pH and the indistinct nature of the Gram smear. In order to limit aeration of the samples those of 5–25 ml. were collected in McCartney bottles, larger samples in flat medicine bottles and the screw caps were rapidly replaced. The samples were then immersed in water in a

thermos flask at 37°–39° before being carried to the laboratory. Stomach tube samples were also taken from those ewes that were not fitted with rumen cannulae.

Samples from lambs were taken 4 hr. and from calves 4½ hr. after the morning feed, usually at weekly intervals. Inoculations were given immediately after the sample had been taken, it being assumed that ciliate organisms present after one week had become established and that any which had survived but not multiplied after the previous inoculation would have disappeared within the week.

Occasionally whole rumen liquor was used as inoculum but in general a concentrated mixed inoculum containing most of the ciliates and many of the bacteria from 500 ml. of rumen liquor was used for each calf and the organisms from 250 ml. for each lamb. Rumen liquor from the rumen cannulated sheep was used (see p. 564). The inoculum was obtained by sedimentation in a separating funnel to which 0·5 % (w/v) galactose was added (Eadie & Oxford, 1955) and the base layer from the funnel was used directly. Where closely comparable inocula were necessary every effort was made to use samples from animals on the same ration and with populations of ciliates similar in both types and numbers. In addition organisms from a concentrate-fed sheep were used to inoculate an animal on that type of ration.

Examination of samples

The pH of each sample was determined with a meter as soon as possible. A pH below 6·0 was considered to be 'low' and above 6·5 to be 'high'. Gram smears and direct microscopic examinations of warmed wet mounts were made from all samples and a small volume of the rumen liquor was fixed with an equal volume of 10 % (v/v) formalin and stored in a sealed bottle.

For the variations of microbial population under consideration thorough microscopic examination of samples under uniform conditions was believed to give as accurate an assessment as attempts to count the organisms. Accordingly for each sample wet preparations were made with standard slides and coverslips. The same magnifications and the same volume of the well mixed samples were used throughout. These examinations could be repeated on the fixed material as and when samples from comparable animals became available.

General observations

The general performance and weekly weights of the isolated ciliate-free and control identical twin calves were recorded and the isolated ciliate-free lambs and their controls were also compared.

RESULTS AND DISCUSSION

Although relatively few of the animals from Tables 1, 2 and 3 are actually cited in the results, generalizations are based on results of the examination of all animals. Thus where a particular treatment is not specifically mentioned it can be assumed that there was no observed effect.

Time of first development of ciliate protozoa

(*a*) *Lambs*

There was very little difference between the times of arrival of ciliates in lambs on the various treatments provided that the ration favoured a fairly high rumen pH.

Ciliates could be present as early as 9 days, were in most lambs by 14 days and were quite definite by 21 days, which is regarded as the stage at which rumination begins (Walker, 1959). There were only scattered fragments of solid food material in the rumen before that age. There was a tendency for an earlier appearance of the ciliates in the outdoor lambs but this was probably due to the favourable ration rather than the presence of the ewe. The establishment of ciliates in the indoor lambs with the ewe seemed to be largely governed by the percentage of concentrates in the ration which in turn affected the rumen pH (see Pounden & Hibbs, 1948*b*, for similar observations on calves).

In general, if the pH was lower than 6·0 only small numbers of ciliates were likely to be present. At a little above pH 6·0, various species of Entodinium were most frequent and at a pH greater than 6·5, a mixed population including the larger Ophryoscolecids developed. In several lambs the large Ophryoscolecids became established before the holotrichs. This ciliate development was irrespective of the source of the ciliate inoculum and despite the fact that a lamb with the ewe was presumably constantly being inoculated by direct contact whereas the artificial inoculations were only at weekly intervals.

(*b*) *Calves*

The first development of a ciliate population in young calves followed a very similar pattern to that in lambs although there was a tendency for Isotricha species to be more rapidly established in calves. Ciliates that had been inoculated into early weaned calves at 8 days old were quite frequently established by the 15th day and this was often a mixed population. However, invariably a change from milk or roughage to a high concentrate ration, with the resultant drop in rumen pH, led to the disappearance of ciliates. The calves 612 and 613 weaned on to a ration consisting entirely of concentrates did not develop ciliates while observations were carried on, which was up to 5 months of age. The use of milk feeding by teat encouraged the maintenance of a high pH in the young calf rumen, presumably by the reduction of overspill of milk into the rumen, and this stimulated early development of ciliates in calves 71, 81 (Table 2) and A (Table 3). However, whatever the method of milk feeding, when concentrates were fed the rumen pH dropped and ciliates disappeared.

When calves were weaned on to diets with certain ratios of roughage to concentrates (Table 2 (*d*)) the rumen pH could be kept above 6·0 and the inoculum of ciliates given at 8 days could be maintained. However, care had to be taken that the ration was adequate to maintain the calves. Calves 5 and 7 developed a thriving mixed rumen ciliate population on a 2:1 and 3:1 roughage to concentrate ration respectively, but became very weak so that the ration had to be changed (Eadie, 1959). A mixture of equal quantities of roughage to concentrates has been found to maintain both calf and ciliates, especially Entodinium species and Isotricha species in the first few weeks.

Establishment of ciliates after a period of low rumen pH

In both calves and lambs the first ciliates to develop after a prolonged period of low pH were Entodinium species. In calves Isotricha species became established very shortly after the entodinia and the large Ophryoscolecids were the last to develop.

This contrasted with the tendency after the same type of inoculum, for a mixed population to develop at once in the young calf where the rumen pH had never dropped below 6·5. When the rumen pH of a calf in which a mixed ciliate population was already established dropped, then Entodinia and Isotricha species were the last to go.

In the majority of lambs which were examined after a period of low rumen pH, the holotrich organisms tended to develop later than the Ophryoscolecids.

The loss of, or more frequently a great reduction in numbers of, ciliates, with a drop in rumen pH occurred in older animals in much the same way as in young ones.

Establishment of ciliates after a period of isolation

(*a*) *Lambs*

The isolation of the lambs 95, 96, 221 and 222 proved very satisfactory. As mentioned by Eadie & Hobson (1962), very small numbers of small entodinia appeared in lamb 95 for 3 weeks but did not become established. They most probably were transferred in droplets from calf *G* which was only a short distance away (see below). Otherwise, no ciliates were ever seen in the isolated lambs until they were inoculated.

Apart from the remarkable failure of these small entodinia to multiply in lamb 95, no difficulty has been encountered in establishing ciliates in lambs that have grown to a considerable age without them on a ration suited to their establishment. When a mixed inoculum was given to lamb 96 at 20 months, a thriving mixed population had developed after 6 days and in this case entodinia and holotrichs were the first to become established. Similarly, when lamb 222 was inoculated with a limited number of ciliate species taken from the isolated sheep in turn, it took only a short time for each species to develop. In this lamb holotrich species were slower to develop than the other species.

(*b*) *Calves*

At about 50 weeks of age small entodinia appeared in both calf *O* and calf *G* and this despite the strict precautions taken to prevent cross contamination. Although calf *G* was kept for a further 11 weeks in the same pen no larger organisms developed. It seems possible that the small entodinia can be carried in droplets and that calf *G* was contaminated by calf *F* which was tethered in its pen only 8 ft. behind calf *G*. Similarly, a year previously when calf *O* became contaminated, calves *N* and *O* were in the same neighbouring pens used by *F* and *G*. It seems therefore, that in isolating calves to prevent rumen ciliate development the distance between animals should be greater than that used in this work and incidentally greater than that required between isolated sheep.

When calves *Y* and *Q* were inoculated, the proportion of concentrates in the ration was still fairly high and only entodinia and isotrichs developed.

After small entodinia had become established by contamination in calf *O* at 23 weeks of age, it was turned out into a field along with its twin *N* and other calves so that it became inoculated by direct contact. The rumen pH of calf *O* at this time was 6·6–7·0. In two weeks a large mixed population of Type B Ophryoscolecids was present along with entodinia but it was 3 weeks later before Dasytricha and Isotricha species were present in fair numbers. This observation is completely com-

parable with the order of development of ciliates under the same conditions, observed by Bryant *et al.* (1958). Similarly, in calf *G* there was a time lag in the development of the holotrichs although there was a large proportion of these organisms in the inoculum.

It appears, therefore, that irrespective of the numbers in the inocula an early development of Isotricha in both very young and older ciliate-free calves is favoured by fairly acid rumen conditions (a pH of 6·0–6·5) such as are found when the concentrate level of the ration is high and that otherwise the Ophryoscolecid population increases more rapidly than the holotrichs.

Effect of the absence of rumen ciliates

There was no significant difference in weight gain, food intake or general performance between animals with and without ciliates and this even in calves *F* and *G* subject to the stress of a low energy diet and in calves *N* and *O* put suddenly out to grass. The observation made by Pounden & Hibbs (1950) of a rougher coat and more pot-bellied appearance in cilate-free calves was confirmed.

Development of flagellate protozoa

In both young calves and lambs, including isolated animals, when the pH was greater than 6·5 and there were few ciliates, a population of flagellates, particularly *Trichomonas ruminantium* Braune with smaller numbers of *Chilomastix frontalis* Braune, developed and flagellates were frequently observed in calves as early as 6 days. This agrees with the observations of Bryant & Small (1960) and Lengemann & Allen (1959). When ciliates became established the flagellates were reduced in number. As observed by Becker & Everett (1930) direct contact is unnecessary for flagellate transfer since in the isolated ciliate-free calves and lambs the numbers of flagellates became exceptionally high and remained high in the adult animal unless the pH within the rumen dropped. These organisms seemed as intolerant of low pH as the ciliates.

The establishment of bacteria

(*a*) *Lambs*

In lambs a Gram smear picture of an 'adult' type, that is one in which there was a conspicuous background of small Gram-negative cocci, coccobacilli and rods, could become established as early as 15 days when the rumen conditions were not acid. This contrasted strikingly with the smear picture from rumen contents with a low pH where large Gram-positive and negative-rods, probably lactobacilli, were predominant and the background of small negative bacteria was inconspicuous if present at all.

Though Pounden & Hibbs (1950) believed that both Oscillospira and the so-called 'window pane' sarcina referred to as Lampropedia by Hungate (1960), could only be transferred by direct contact between ruminants in the same way as rumen ciliate protozoa, both these organisms developed in isolated ciliate-free lambs. The 'window pane' sarcina was never present in large numbers in any lambs, but Oscillospira in numbers over 10^5/ml. was frequent and this organism reached particularly high numbers in the isolated lambs (Eadie & Hobson, 1962). Quin's ovals and selenomonads also developed in considerable numbers in faunated and ciliate-free lambs. (See Moir & Masson (1952) for illustrations of these organisms.)

The first of these types of bacteria to be seen were invariably small selenomonads. These were followed by Quin's ovals and large selenomonads while Oscillospira was always the last to become established.

Table 4. *Age of lambs (in days) when small selenomonads and Oscillospira were first observed in rumen samples taken weekly*

Lamb no.	Small Selenomonads	Oscillospira
305	53	104
327	56	78
90	28	56
92	27	55
94	23	37
91	Between 22 and 57*	78
93	35	84
95	36	50
96	49	126
88	49	70
89	50	99
411	35	56
418	None seen by 88	None seen by 88
221	Between 49 and 63*	70
222	49	63
230	32	39
231	25	54
232	28	70
241	30	None seen by 44
242	None seen by 44	None seen by 44
245	35	None seen by 44
246	None seen by 44	None seen by 44
247	None seen by 44	None seen by 44

* The intervening samples were abomasal contents.

Table 4 illustrates the consistent order of arrival of organisms as well as certain variations between animals. Lambs 418, 241, 242, 245, 246 and 247 are of interest. In the first 3 weeks a mixed ciliate population had developed in several of these lambs but later they consumed considerable quantities of the ewe's concentrates. At the stage when small selenomonads were seen in lambs 241 and 245 these were the only two of this group in which a few ciliates, entodinia, remained. By 44 days all these lambs appeared to be ciliate-free, the rumen pH had dropped below 6·0 and small selenomonads were no longer visible. Thus the difference between the twins 241 and 242, 245 and 246 could be accounted for by difference in rumen pH caused by a variation in concentrate consumption. However, the striking lag between the arrival of Oscillospira in the isolated ciliate-free lambs 95 and 96 cannot be so easily explained. At this stage both lambs were together in one pen and it would seem that lamb 96 had every opportunity for direct transfer of Oscillospira from lamb 95 in addition to the same opportunity for the more remote transfer effective in lamb 95. Moreover, there was no obvious difference in the rumen contents of 95 and 96 which might account for the 10 week lag, and 1 week after the organism was first observed in 96, it had reached very high numbers.

Similarly the difference of one month in the times of arrival of Oscillospira in lambs 88 and 89 was unexpected. These lambs were fed together from 44 days and lamb 89 had had a mixed ciliate population from 43 days. Thus, if Oscillospira

first develops under conditions suited to ciliate establishment, as seemed possible from earlier results, it would have been expected much earlier in lamb 89. It should be noted that the reduction in numbers of Oscillospira after the establishment of ciliates in isolated lamb 96 (Eadie & Hobson, 1962) was a reduction to numbers comparable with those in a faunated animal on the same ration and did not indicate competition which would lead to the complete exclusion of Oscillospira.

Large numbers of a motile oval organism up to 12μ in length were occasionally observed in the isolated lambs (Pl. 1, figs. 2, 3). Locomotory organelles were never visible. This organism has so far not been identified but it does not appear to correspond to any rumen organism so far described in the literature.

(*b*) *Calves*

Like the lambs, the lactobacillus Gram smear picture in calves with a rumen of low pH was very distinct from the 'adult' picture. The small organisms of the 'adult' picture appeared to be very similar to those in lambs or sheep. There was no difference between calf 86 and the calves of group (*a*) Table 2, so that as in Rusoff & Davis' observations (1951), aureomycin did not have a striking effect on the flora.

There was one distinct contrast between the observations in calves and lambs: the large bacteria—Selenomonas species Quin's ovals and Oscillospira—were never conspicuous in calves, even though they were invariably present in the routine rumen inocula which were taken from sheep. Even an inoculum of whole rumen liquor from an isolated lamb, in which were very high numbers of Oscillospira, failed to establish this organism in calf *F*. The only occasions when Oscillospira persisted, and then only in small numbers, were in calf *O* and to a lesser extent in calf *N* after these animals had been in contact with other calves in a field. In calf *O* small numbers of Oscillospira were present for 4 months.

It is difficult to explain the lack of 'large bacteria' in the calves when in every other respect the lamb and calf bacterial pictures were very similar. The low numbers of Oscillospira in isolated calves are especially remarkable since Pounden & Hibbs (1950) when working with an isolated ciliate-free calf which had been accidentally contaminated with normal calf rumen fluid, observed vast numbers of Oscillospira, an observation entirely comparable with those recorded for the isolated lambs discussed above. Although calf *Z* was kept for 8 weeks in a crate about 9 ft. away from some adult roughage-fed cattle and the dairy herd, and might be expected to have been subject to aerial contamination with Oscillospira, none of these organisms developed in the calf. Even when calf *Z* was directly inoculated with 100 ml of whole rumen contents from isolated lambs on a similar ration but containing many Oscillospira, the organisms failed to develop. A very few organisms, circa 20/ml., were observed during the first 3 days following the inoculation but they completely disappeared after this.

It would seem, therefore, that some feature of the calf rumen fluid was unsuited to the development of Oscillospira. As with other calves neither Quin's ovals nor selenomonads developed in calf *Z*. The 'window pane' organisms were more frequent and more numerous in calves than in lambs.

Inter-relationships between bacteria and ciliate protozoa

Except in the early-weaned calves 3 and 4 (Eadie *et al.* 1959), the only observations of the 'small bacteria' in the present work were from direct films so that generalizations on the flora can be made only with care. However, the observations on the relationship between the number of lactobacilli which was controlled by diet, and ciliate establishment were quite general. Also, where ciliates were present in abundance the normal 'adult' type Gram-negative background to the film was invariably present and this even in the case of very young calves where a mixed ciliate population was present only for a few days. A number of workers, including Pounden & Hibbs (1948*a*), and Huhtanen, Saunders & Gall (1951), have noted differences in flora between normal young calves and mature animals. From the present work it appears that it is ration rather than age which determines the predominant bacteria in exactly the same way as it controls the ciliate development, and the 'adult' bacterial picture is normally associated with ciliates and a high rumen pH.

The presence of a significantly greater number of bacteria in the isolated ciliate-free lambs compared with the control lambs with ciliates, has been discussed (Eadie & Hobson, 1962). On inoculation with ciliates the total number of bacteria in isolated lamb 96 dropped considerably and the same was clearly true for lamb 222 although counts were not made. However, the morphological types of bacteria in isolated ciliate-free lambs and calves made up an 'adult type' population which did not differ markedly from that in the inoculated controls, except for a distinctive, large, parallel-sided, granular-staining, Gram-positive rod (Pl. 1, fig. 1) which was present in considerable numbers for a period of time in all ciliate-free animals on a high roughage diet. This organism did not remain permanently in isolated animals but it was observed as early as 5 weeks in several lambs and it stayed for 5 months in one case where isolation was prolonged. It was never observed in animals with rumen ciliates, nor was it seen under ciliate-free, acid ruminal conditions. In calves 610 and 611 which had been kept free from ciliates initially because of the high-concentrate weaning ration and consequent low rumen pH, this characteristic rod developed during a short period of roughage feeding. As the isolated animals were kept at different times and in different houses, it seems that the conditions cited above, namely the ciliate-free rumen of a roughage-fed young animal, are particularly favourable for this organism. Pounden (personal communication) has noted a Gram-positive granular rod associated with isolated calves but it is impossible to compare the organisms without cultural observations.

The components of the ciliate population

As mentioned earlier, a mixture of Entodinium species, holotrichs and large Ophryoscolecid organisms could develop in the very young animal and a mixed population is typical of the normal domestic or wild ruminant except that the holotrichs are sometimes absent. The only Ophryoscolecid which always failed to become established when inoculated into the young animals, was *Ophryoscolex tricoronatus*. It was frequently present in the inocula for lambs 51, 52, 305, 327 and calves *X* and *P*. When conditions in the rumen had been acid entodinia always appeared first in lambs. However, in general where a lamb was with the ewe a mixed population

developed which represented in the number of species and their relative proportions that found in the parent animal. Thus, the population was that which might be expected to develop after direct contact. However, the relationship between the ciliates which developed in the inoculated lambs and calves and those present in the artificial inocula, was more complex and will be discussed in detail by Eadie (1962).

GENERAL CONCLUSIONS

It has been established that both lambs and calves can develop a mixed ciliate population at a very early stage given a high pH in the rumen, and that the degree of acidity takes precedence over the source of inoculum or the age of the animal in governing the development of both a mixed ciliate fauna and an 'adult' type flora.

The general pictures for the establishment of ciliates in lambs and calves were very similar and comparable, as were the bacterial pictures, except that very few Quin's ovals, selenomonads and Oscillospira ever developed in calves. Direct contact between animals has been proved to be unnecessary for the transfer of these bacteria. A characteristic large Gram-positive granular rod was noted to be present for some time in all roughage fed, ciliate-free animals. In neither calves nor lambs (see Eadie & Hobson, 1962) was there any significant difference in general performance between animals maintained with and without ciliates. It seems possible that small ciliates can be dispersed further from cattle than from sheep and that this has to be taken into account when isolating the animals in order to keep them ciliate-free.

Despite these generalizations made from observations on 58 animals there still remained certain individual variations between animals which could not be accounted for by difference in management and this type of variation has been noted by previous workers (Bryant & Burkey, 1953; Bryant & Small, 1960). This stresses the importance of basing conclusions on a sufficiently large number of animals with additional controls kept at different times. The general picture for a given treatment has proved to be similar but the actual arrival times and comparative numbers of various organisms varied considerably and presumably indicated certain internal conditions of the host which had to be attained before establishment was possible. Probably the acid rumen associated with a high concentrate ration is the only condition in which the organisms can be relied upon to develop as predicted.

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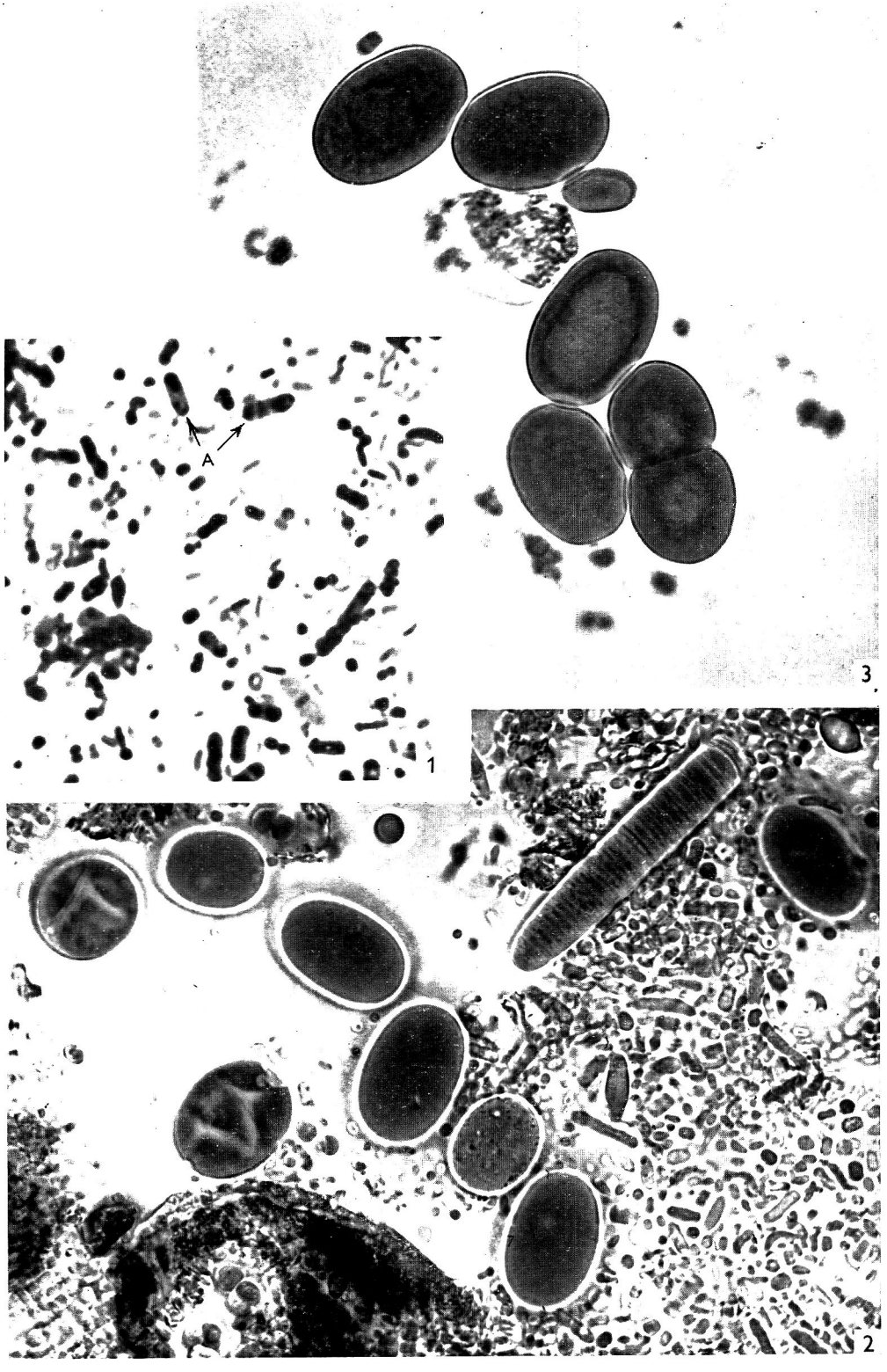
EXPLANATION OF PLATE 1

Fig. 1. Large granular-staining Gram-positive rod from an isolated calf. 'A' indicates two particularly typical organisms (× 2,300).

Fig. 2. Large oval organism from isolated lamb with normal bacterial background and one Oscillospira organism. Direct U.V. illumination, λ 2536 A. (× 2240).

Fig. 3. Large oval organism partially separated from debris and showing some visible internal structure. One dividing organism is shown. Direct U.V. illumination (× 2240).



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Inter-Relationships between Certain Rumen Ciliate Protozoa

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SUMMARY

The establishment and components of the rumen ciliate population in a series of young animals has been followed using intra-ruminal inoculation with rumen material, and for comparison purposes the ciliate population in a number of adult ruminants has been examined. The fact that the ciliates *Polyplastron multivesiculatum*, *Eudiplodinium maggii* and *Epidinium* spp., though not host specific, did not form a stable mixed population was noted and experiments were carried out to examine the antogonism between certain of these organisms. The cause of the antagonism was not determined but cannibalism, food competition, or gross bacterial change did not seem to be responsible. It was found that the population of an adult animal could be changed by inoculation but the relationship between the ciliates appeared to be in some way affected by the host. It is concluded that inter-relationships of the type described may play an important role in determining the components of a particular rumen microfauna.

INTRODUCTION

The present paper will discuss in more detail the components of the ciliate populations in the series of young calves and lambs examined by Eadie (1962).

The presence of a certain degree of host specificity in rumen ciliates has been noted (see Dogiel, 1947). However, the ciliates used in the present investigation are those which are found naturally in both sheep and cattle.

In cannulated sheep (Eadie, 1962) the ciliate population tended to be a mixture of *Entodinium* spp., holotrich species and either type A large Ophryoscolecids, *Polyplastron multivesiculatum* (Dogiel & Fedorowa) with various other species such as *Diploplastron affine* (Dogiel & Fedorowa) and *Ophryoscolex tricoronatus* (Dogiel), or type B in which *Eudiplodinium maggii* (Fiorentini) and *Epidinium* spp., either together or alone, were the predominant large Ophryoscolecids. It became clear in the course of the work with artificial inoculations that the two types appeared to be unable to form a stable mixed population in sheep or cattle. Difficulties in establishing rumen bacterial species have been encountered by Hobson & Mann (1961). This paper discusses the initial observations of antagonisms between rumen ciliates and describes further experiments carried out to investigate this problem.

METHODS

Animals

Tables 1 and 2 list most of the animals used in this work. Two kids were fed a ration similar to the lambs and were regularly inoculated with mixed rumen ciliates. The rations and management of the other young animals have already been

recorded (Tables 1, 2 and 3, Eadie, 1962). 43 and 74 were rumen-cannulated wethers.

Rumen contents were also examined from cattle and goats at this Institute, from red deer killed near Braemar, Aberdeenshire, and on the Isle of Rhum and fixed samples of rumen material from Zebu and Hereford cattle from Kenya were also used.

Stomach tube sampling and inoculation and the examination of samples

The routine procedure was that described by Eadie (1962). Where the inoculum was to contain a mixture of two possibly antagonistic populations the same separating procedure was used but the rumen liquors containing each type were kept apart until just prior to filling the McCartney bottle. The mixed inoculum used for lamb 232, for example, came from the same funnels and was therefore exactly comparable to the separate control inocula of types A and B given to lambs 230 and 231, respectively.

When a change in population was thought to be imminent samples of at least 25 ml. were taken every 1–3 days. Before an organism was considered to be absent a survey had been made of all organisms in up to 2 ml. of whole rumen liquor in 0·1 ml. drops followed by examination of drops of fixed and concentrated material. Since the organisms were large and easily seen a negative result indicates that no organisms had been observed amongst ciliates from at least 5 ml. of rumen fluid as compared with thousands/ml. in an average population.

Identification of the ciliates

Without discussing in detail the controversial problem of classification it seems, nevertheless, essential that it is quite clear to which organisms this work refers. The problem of nomenclature, particularly for the organism *Polyplastron multivesiculatum* and even more so for the closely related *Elytroplastron* sp. has taken up much space in the literature, notably the papers of Becker (1933) and Wertheim (1935).

It should be emphasized that the author is completely satisfied that all the organisms referred to in this work by one particular specific name were of the same species. It was considerably more difficult in the case of *Polyplastron multivesiculatum* to determine what that species was. However, after careful examination of organisms from a sheep starved for 30 hr. and comparison with fixed specimens of *P. multivesiculatum* kindly sent from France by Dr C. Noirot-Timothee the presence of a small central dorsal skeletal plate was finally confirmed and the organism identified as *P. multivesiculatum*. The three dorsal skeletal plates were not as sharply defined as Dogiel & Fedorowa's diagram (1925) would suggest and as observed by Noirot-Timothee (1960) they were not joined by material of a skeletal plate nature so that the 'M' shape mentioned by Kofoid & MacLennan (1932) is misleading. After re-examination of preserved specimens and consultation with Dr B. Sugden it has been concluded that organisms previously recorded as *Metadinium medium*, Awerinzew and Mutafowa (Sugden, 1953; Eadie, 1957) and those described by Eadie (1957) as *Elytroplastron bubali* (Dogiel) were all in fact *P. multivesiculatum*.

In order to remain relatively consistent Kofoid & MacLennan's classification

rather than that of Dogiel has been used and where Dogiel's work is quoted the names used by Kofoid & MacLennan have been substituted. The nomenclature used is therefore that of Lubinsky (1957) but differs from Noirot-Timothee (1960) in retaining the generic names *Eremoplastron*, *Diploplastron*, *Metadinium* and, particularly convenient for this work, retaining only *Eudiplodinium maggii* within the genus *Eudiplodinium*. This scheme has been chosen in order to describe the present work more easily rather than to express a theory of rumen ciliate systematics.

RESULTS

As previously noted (Eadie, 1962) both type A and B populations were found in different sheep although type A tended to be the most prevalent. In a number of mature cattle examined type B was most frequently present but type A was not unknown. Ophryoscolex was not present in the sheep with type A which were used in this work, except in that used to provide the inocula for the two kids.

Tables 1 and 2 give a condensed summary of the following observations.

Table 1. *Observations on antagonism between rumen ciliate species in lambs and sheep*

Animal	Initial population (if any)	Inoculum	Changes	Final population
Sheep				
43	Type B (Epidinium + Eudiplodinium)	Chance contact, type A	Rapid loss, type B	Type A
74	(1) Type B (Epidinium)	Type B (Epidinium + Eudiplodinium)	Eudiplodinium established	—
	(2) Type B (Epidinium + Eudiplodinium)	Type A	All type B disappeared	Type A
Lambs				
89	Type B (Epidinium + Eudiplodinium)	Type A	All type B disappeared	Type A
222	(1) —	Entodinium + Dasytricha from partially faunated sheep	Entodinium + Dasytricha	—
	(2) Entodinium + Dasytricha	Type B (Epidinium) from partially faunated sheep	Type B (Epidinium) established	—
	(3) Type B (Epidinium)	Type A (Polyplastron + Isotricha) from partially faunated sheep	All type B disappeared	Type A
230	—	Type A	Type A established	Type A
231	(1) —	Type B	Type B established	—
	(2) Type B	Contact with 230 and 232 continued	All type B disappeared	Type A
232	—	Mixed types A and B	Type A only seen	Type A

(*a*) *Sheep and lambs.* The first clear indication of antagonism was shown when the 18-month-old wether 43 which had had a steady population of type B organisms for at least 3 months, suddenly overnight 'lost' Epidinium and Eudiplodinium organisms and within 2 days contained large numbers of *Polyplastron multivesiculatum*, i.e. type A. The entodinia and holotrichs were unaffected. This animal was in a pen within contact with at least one sheep which contained type A organisms. The type A population has not changed again after 2 years.

Next the 2-year-old wether 74 in which a type B population of *Epidinium* spp. but no Eudiplodinium, had been naturally established for at least 7 months, was inoculated with rumen liquor from a sheep containing a mixed type B population of *Eudiplodinium maggii* and Epidinium spp. Within 4 days Eudiplodinium was seen in sheep 74. One week later 400 ml. of rumen liquor containing type A organisms was inoculated directly into the rumen cannula and the following day a few Polyplastron were seen. After 7 days a fair number of type B organisms were still present along with Polyplastron but by the 11th day only type A organisms

Table 2. *Observations on antagonism between rumen ciliate species in calves*

Animal	Initial population (if any)	Inoculum	Changes	Final population
Calves				
3	(1) —	Type A alone	Low pH—only Entodinium spp. and holotrichs established	—
	(2) Entodinium + holotrichs	Mixture of types A and B	Type B seen first then mixture and finally A	Type A
4	(1) —	Type A alone	Type A established	—
	(2) Type A	Mixture of types A and B	Type A *only* seen	Type A
35	(1) —	Mixture of types A and B (Epidinium)	Type B (Epidinium)	—
	(2) Type B (Epidinium)	Mixture of types A and B (Epidinium + Eudiplodinium) alternated with type A alone	Type B (Eudiplodinium + Epidinium)	Type B
'N'	(1) —	Inoculated type A	Type A	—
	(2) Type A	Contact with animals with type B and cross-inoculation from 'O'	First type B but finally A only	Type A
'O'	(1) Small Entodinium spp.	Contact mature animals	Mixed type B	—
	(2) Mixed type B (i.e. including Ostracodinium spp. & Eremoplastron spp.	Cross-inoculation from 'N'	Type B disappeared	Type A
'F'	(1) Short time type B (Eudiplodinium)	Type A	Type A established	—
	(2) Type A	Series inoculations of alternate types A and B	Type B (Eudiplodinium) stayed 5 weeks	—
	(3) Type B (Eudiplodinium)	Left without inocula	Wavered considerably and mixed for periods. Finally type B (Eudiplodinium)	Type B
'G'	—	Inoculated type B	Type B (Eudiplodinium) established	—
	Type B (Eudiplodinium)	Inoculated alternately with types A and B	Type B (Eudiplodinium) remained	Type B

remained, although not in large numbers. Attempts were made to re-establish type B organisms by inoculation of 500 ml. volumes of whole rumen liquor from the sheep which had earlier been used to establish Eudiplodinium (see above), but the change to type A seemed to be irreversible and remains so. It is of interest that no type B organisms were ever observed even transitorily, after the inoculations, in contrast to certain observations in experiments with calves (see later).

A further experiment was carried out with lamb 89 in which a thriving type B population had been established by direct contact with the ewe. At 4 months of age it was inoculated with whole rumen liquor of type A and within one week all type B organisms had disappeared and only type A remained. Again the change proved irreversible, which showed with certainty that this was a feature of the ciliates and bore no relation to the original inoculum given to the lamb. The early history of sheep 43 and 74 was not known so that the change might have been a reversion to an earlier established population. However the fact that type B had been the only population in lamb 89 since birth ruled out this possibility.

Having proved that an established type B population in sheep could be changed by inoculation with type A, even if the inoculum was quite small as must have been the case in the natural inoculations of sheep 43, it became of interest to determine if a mixture of populations could be achieved when both types were given an equal opportunity to become established in a young lamb. For this the mixed inoculum prepared as described under 'Methods' was used for lamb 232 from 18 days old. Control lambs 230 and 231 were inoculated with types A and B populations, respectively, and all three lambs were kept together in one pen. It was of interest that holotrichs were absent from the type B inoculum used. When inoculation was terminated at 46 days old, lamb 230 had a type A population and lamb 231 had type B as inoculated, i.e. without holotrichs. This proved that both populations were perfectly viable. However, lamb 232 had only a type A population and no organisms of type B were ever seen. The lambs were kept together and the populations remained unchanged up to 115 days old. On that day holotrichs were first seen in lamb 231 which proved that there had been transference from 230 or 232. One week later the previously thriving population of type B organisms had completely disappeared from lamb 231 and type A alone was present. Once again there was no reversion.

The final experiment with sheep was carried out on lamb 222 after isolation (see Eadie, 1962). This was partly to confirm that isolation did not alter the relationships between ciliates and that the antagonism was found irrespective of the number of ciliate species present. When *Entodinium* spp., *Dasytricha* sp. and *Epidinium* spp. had been established by inoculations from a partially defaunated sheep containing these species alone (Eadie, 1962), a population of type A from another isolated sheep containing only *Polyplastron multivesiculatum*, *Entodinium* spp. and *Isotricha* spp. was inoculated into lamb 222. Both Epidinium (type B) and Polyplastron (type A) were seen for 5 days but on the 6th day the only large Ophryoscolecid which remained was Polyplastron (type A). In this sheep the holotrich organisms were rather slow to increase in number so that, in fact, at the time of change other than Polyplastron and Epidinium, only entodinia species were present in any significant numbers. This experiment therefore confirmed a direct relationship between the Polyplastron of type A population and Epidinium of type B.

(*b*) *Calves.* From Table 2 it can be seen that experiments similar to those in the lambs were carried out with calves.

In the young calves, 3, 4 and 35, a mixed inoculum was used. In calf 4 the type A population alone persisted though on one day a mixture was seen. In calf 3 type B first became established but was later replaced by type A. In calf 35, however, none of the type A Ophryoscolecids were ever seen but type B became established

from 56 days of age and even where large inocula from sheep containing type A were given, type B remained in this calf. These results show a contrast between lambs and calves in that type A had always been completely dominant in sheep.

In the three experiments described above the inocula were all taken from sheep. Observations on the antagonism were also made on calves 'N' and 'O' where 'N' had initially been inoculated with type A from sheep but relatively few ciliates had developed. Calf 'O' developed a mixed type B population by direct contact with other calves when both calves were turned out to grass at 164 days and inoculations were stopped. This population in 'O' included *Ostracodinium* spp. and *Eremoplastron* spp. as subsidiary type B organisms. Gradually, while still in contact with other calves, the type A organisms in calf 'N' disappeared and type B organisms developed and were quite numerous 7 weeks after calf 'O"s population had been established. One week later, however, the population in calf 'N' had reverted to type A.

At this stage the calves 'N' with type A organisms and 'O' with type B organisms were cross-inoculated several times with up to 150 ml. of whole rumen liquor. Calf 'N' showed only type A organisms throughout. Calf 'O' began to have a few Polyplastron (A) and 3 weeks after the first cross-inoculation only type A remained and all type B, including the subsidiary species, had disappeared. Cross-inoculation was stopped but type A remained stable in both calves for the next 2 months at which stage sampling was stopped.

Finally, twin calves 'F' and 'G' were used in antagonism investigations. Calf 'F' following an initial period with type B, developed type A after inocula of this type had been given regularly. Calf 'G', after isolation for 9 months, was inoculated with type B which became rapidly established. A series of alternate inoculations with types A and B in turn, were then made to both calves and an interval of up to 4 weeks was left between inoculations. Despite the large volumes of inocula of type A, type B persisted in calf 'G' and no organisms of type A were ever seen. In calf 'F', on the other hand, the population wavered considerably and at the time the last inoculation of type A was given Eudiplodinium (type B) had persisted for 5 weeks though Epidinium (B) had failed to establish even after large numbers were given from the partially defaunated sheep containing that organism.

Three weeks after the final inoculation of calf 'F' with type A, type B was still the only one seen. However, during the next 4 months when no inocula were being given and the animals were still being kept apart, the population again wavered. Polyplastron (type A) developed along with type B Eudiplodinium organisms for a period of 2 weeks and type A was then present alone for a further 2 weeks. There followed another short time with the mixture and finally type B became predominant and type A disappeared. This was the only occasion on which Polyplastron (type A), having once been observed in either a calf or a lamb, did not become predominant and it is particularly remarkable in that for several weeks it had virtually been the only large Ophryoscolecid.

General observations on the antagonism

(*a*) There was no change in the bacterial Gram smear picture to coincide with alterations in ciliate population.

(*b*) The frequent presence within the protozoa of parasites of the genus *Sphaerita* described by Lubinsky (1955) appeared to bear no relation to the antagonism.

(*c*) Cannibalism was not observed.

(*d*) The rations fed differed (Eadie, 1962) so that a particular food material was not significant.

(*e*) Since on occasion a large number of type B organisms were replaced by relatively few type A organisms direct competition for food seemed unlikely.

(*f*) Neither entodinia nor the holotrich ciliates were involved in the changes.

(*g*) Whether Epidinium and Eudiplodinium were together or alone in a type B population seemed immaterial and from calf 'O' it appeared that other species could be involved in a type B population.

(*h*) *Diploplastron affine* tended to be associated with type A only. The position of Ophryoscolex is discussed later.

(*i*) When the population in calf 'F' included both types some unusually large Eudiplodinium organisms were observed. Some organisms were dividing and were, therefore, larger but the width was up to 40 μ greater than the largest from a comparable 'normal' population. The sizes did not correspond to the large and small organisms described by Fiorentini (1889) as *E. maggii* and *Diplodinium bursa*.

Observations of ciliate populations from other hosts and at other times

Eudiplodinium maggii and *Epidinium* spp. can exist with some other large Ophryoscolecid species since they were observed along with organisms of the genus *Metadinium* in deer and in Hereford and Zebu cattle from Kenya and in the latter the largest recorded Ophryoscolecid *Eremoplastron giganteum* (Dogiel) was also present. This species had previously only been recorded from wild ruminants in Africa (Dogiel, 1925).

Polyplastron multivesiculatum has been observed by the present author in association with two other fairly large Ophryoscolecids; with *Enoploplastron triloricatum* (Dogiel) in sheep from North Ronaldsay and with *Ophryoscolex tricoronatus* in several sheep at this Institute. Ophryoscolex could be established with Polyplastron in mature sheep but as mentioned by Eadie (1962) it did not readily become established in either lambs or calves. It developed in one kid at 4 months of age, though in other respects the kids were similar to lambs. With no known stimulus Ophryoscolex died out in the population of one old sheep in spring, 1958 and in two others in late 1959 and it seemed therefore to be less firmly established than Polyplastron.

DISCUSSION

Kofoid & MacLennan (1933) remark upon the tendency for certain species of rumen ciliate to appear in one host 'in preference' to another. This is also clear from Dogiel's observations (Dogiel, 1947). Becker (1932) in referring to this apparent mild form of specificity poses the question of whether this is due to 'unequal distribution due to specifically adapted infusoria or to individual physiological

properties of the various host species'. The observations on antagonism between ciliate species in the present work may partly solve this question of variation in ciliate populations between animals and between host species. Nevertheless, the presence of *Eremoplastron giganteum* (Dogiel) in Zebu cattle from Kenya and not in the neighbouring Hereford animals, might indicate that certain of Dogiel's ideas of host specificity are valid.

Unfortunately in the papers which record the presence of *Polyplastron multivesiculatum* there is no record of the other species found in the same host individual, though Dogiel & Fedorowa-Vinogradova (1925) state that Polyplastron is frequent in cattle and sheep. Wertheim (1935) lists cattle, sheep and goats as the recorded hosts. Dr Noirot-Timothee (private communication) has noted that, though in the majority of samples from sheep and cattle which she has examined types A and B were separate, in only a small number of cases she found Polyplastron in the same population with *Eudiplodinium maggii* and even less frequently with *Epidinium* spp. Unfortunately there were isolated observations and it seems possible that a series of samples from the same animals might have shown that the populations were in the process of changing (see calf 'F'). The cross-inoculation work of Dogiel & Fedorowa-Vinogradova (1930) can more easily be compared with the present findings. Early in their paper the Russian workers listed 19 species which they had recorded from goats and this list included Polyplastron, Epidinium and Eudiplodinium. In their experiments goats partially defaunated by starvation methods were inoculated with rumen contents pooled from several bulls killed at the slaughter house. Although Dogiel & Fedorowa's interest was in the species which established and not in the relationship between the ciliates themselves, several similarities to the results of the present work can be seen. *Eudiplodinium maggii*, Epidinium and Polyplastron never became established together. In one experiment Polyplastron became established in a goat already containing *Diploplastron affine* and *Diplodinium caudatum* and on this occasion the authors noted the temporary presence of small numbers of *E. maggii*, *Eremoplastron* spp., *Ostracodinium* spp. and *Metadinium medium* for 3 days after inoculation after which they 'probably died'. When this animal was re-inoculated Polyplastron disappeared and *M. medium* and Eremoplastron became established. Dogiel & Fedorowa checked that the established species were not necessarily those most numerous in the inocula but they explained the observations on the basis of different susceptibility to the conditions in the goat. It seems clear that they were confronted with the same type of antagonism between ciliates as is described in the present work. Our observations on kids and goats have indicated a strong similarity to sheep.

The specific factors involved in the antagonism are not clear but the fact that it exists is beyond doubt. Although the ciliates concerned display no host specificity a definite host effect has been observed in that type A though dominant in sheep was less so in cattle and in fact, type B displayed dominance in calves 35, 'F' and 'G' under the same conditions. This type of variation might lead to host population differences of the kind observed by Dogiel. The fact that considerable time may elapse before contact effects transfer between animals, as in the case of lamb 231, is rather a surprising observation but it is nevertheless clear that natural contact may be completely effective in altering a population from a non-dominant type. It seems probable that other species of ciliate may show mutual antagonisms, as for

example that between *Metadinium medium* and Polyplastron indicated by Dogiel & Fedorowa's work (1925) and it is hoped to examine this further.

At present it is not possible to correlate antagonism with food competition, cannibalism or gross bacterial population changes nor even with the species regarded as evolutionarily the most complex since Dogiel (1947) and Lubinsky (1957) both place Ophryoscolex at least as high or at a higher level than Polyplastron. Similarly, large size does not in itself seem to be significant since *Eremoplastron giganteum* was associated with Eudiplodinium and Epidinium. The large size of certain Eudiplodinium organisms in the mixed population of calf 'F' has not been explained. The fact that apparently closely related species may not have the same antagonistic properties is illustrated by the records of *Elytroplastron bubali*, since out of the ten buffaloes and goats from which Elytroplastron was obtained in at least seven cases organisms of type A were also found (Dogiel, 1928; Das Gupta, 1935).

The presence of antagonism between rumen ciliates indicates that the initial large ciliate population transferred to the young ruminant from the dam is not only subject to change through alteration in rumen conditions caused by a change in ration (Eadie, 1962) but also through the 'take-over' by an antagonistic species and in order fairly to assess the establishment of a particular ciliate species in a ruminant host it is necessary to consider that species in relation to the rumen ciliate population as a whole. Thus the factors which govern the development of a rumen ciliate fauna are not as straightforward as was first believed.

I am most grateful to Dr B. Sugden and Dr C. Noirot-Timothee for their help in the identification of *Polyplastron multivesiculatum* and to Mr G. Philip for making available the samples from Kenya. I should also like to thank Dr P. N. Hobson for his advice and encouragement and Mr W. Shand for continual technical assistance.

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Lactate and Pyruvate Catabolism in Acetic Acid Bacteria

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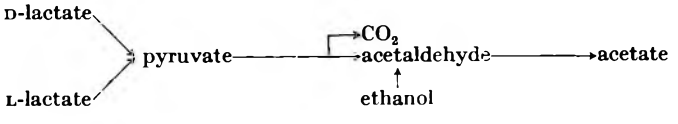
SUMMARY

The enzymic mechanism of D- and L-lactate catabolism was the same in several strains of *Acetobacter* and *Gluconobacter*. This was taken to indicate a close relationship between these groups. Both isomers were broken down by way of pyruvate and acetaldehyde to acetate. Identical reactions were carried out by two sets of enzymes of a different nature and localization, one particulate, the other soluble. All strains contained a constitutive, soluble pyruvate decarboxylase which required thiamine pyrophosphate and Mg^{++}. When grown on lactate, a pyruvate decarboxylase was also present in the particulate fraction. No evidence was found for a pyruvate oxidase system. Acetaldehyde was oxidized by a particulate oxidase system and by NADP- and NAD-linked dehydrogenases. This last enzyme was activated by coenzyme A and glutathione. The particulate pyruvate decarboxylase was not very tightly bound to the particles, as it could easily be detached by washing with buffer. The oxidase systems could not be removed under these conditions.

INTRODUCTION

The 'acetic acid bacteria' are divided into the polarly flagellate *Gluconobacter* (also called *Acetomonas*) and the peritrichous *Acetobacter* (Leifson, 1954; Shimwell, 1958; De Ley, 1961). In spite of this morphological difference both biotypes show many physiological and biochemical similarities, indicating a close relationship. It seemed that an investigation of the comparative metabolism of pyruvate in these bacteria might offer further possibilities in exploring their taxonomic relationship, since three different mechanisms for pyruvate catabolism have been reported. A direct pyruvate decarboxylase was described for *G. suboxydans* (King & Cheldelin, 1954) and for *A. peroxydans* (De Ley & Schell, 1959). A pyruvate oxidase system was reported for *A. pasteurianus* (King, Kawasaki & Cheldelin, 1956) and *A. liquefaciens* (Stouthamer, 1960). According to Rao (1955) in *A. aceti* acetaldehyde was not an intermediate in pyruvate breakdown, nor did lipoic acid play a role. In previous studies (De Ley & Schell, 1959) we have established that subcellular particles from *A. peroxydans* oxidized D- and L-lactate, pyruvate and ethanol with oxygen as terminal electron acceptor. The particles contained pyruvate decarboxylase and a cytochrome system, and no additional co-factors were required. The reactions are summarized in the following diagram:



An identical reaction sequence occurred in the cytoplasm, but with enzymes of a different nature. The soluble D- and L-lactate dehydrogenases required the addition of artificial electron acceptors to demonstrate their activity. The oxidation of ethanol required either NAD or NADP and acetaldehyde dehydrogenase was NADP-linked.

The present paper is concerned with a study of the metabolism of lactate and pyruvate by other 'acetic acid bacteria'.

The following abbreviations will be used: CoA coenzyme A; GSH, reduced glutathione; ThPP, thiamine pyrophosphate; NAD, nicotinamide-adenine dinucleotide; NADP, nicotinamide-adenine dinucleotide phosphate; $NADH_2$, reduced nicotinamide-adenine dinucleotide; $NADPH_2$, reduced nicotinamide-adenine dinucleotide phosphate.

METHODS

Organisms. The origin of the strains used was described previously. We shall adhere here to the nomenclature of strains previously proposed by De Ley (1961). The following strains were investigated: *Acetobacter aceti* strain Ch 31, *A. aceti* (*rancens*) strain 23, *A. aceti* (*pasteurianus*) strain 11, *A. aceti* (*turbidans*) NCIB 6424, *A. aceti* (*xylinum*) strain 25, *A. aceti* (*liquefaciens*) strain 20 (this strain has previously tentatively been classified as a *Gluconobacter*, due to the uncertainty of its flagellation; recent investigation of its infrared spectrum (A. W. Scopes, personal communication) showed that it belongs in the biotype *Acetobacter*), *Gluconobacter oxydans* (*suboxydans*, strain 26; *G. oxydans* (*melanogenus*) NCIB 8086. Other strains of our collection were occasionally used. When received the strains were examined for purity by plating and their diagnostic characteristics redetermined. During the course of the investigation the purity and homogeneity of the strains was occasionally checked by plating.

Growth of organisms. Strains aceti Ch 31, rancens 23 and xylinum 25 were grown in the following lactate medium: M/120 KH_2PO_4, M/120 Na_2HPO_4, 0·2 % yeast extract (Difco), 0·2 % NH_4Cl, 0·025 % $MgSO_4.7H_2O$, 2 % ammonium DL-lactate and 0·02 mg. % bromocresolpurple, pH 6·5. The organisms were grown and harvested as previously described (De Ley & Schell, 1959). Strain pasteurianus 11 did not noticeably grow on this medium, in spite of the fact that lactate was consumed. Therefore this strain, as well as aceti Ch 31, rancens 23, turbidans 6424, suboxydans 26 and melanogenus 8086 were grown on a glycerol medium, which had the same composition as above, except that 2 % glycerol was substituted for DL-lactate. Other strains were grown in Roux flasks on solid media containing 1 % yeast extract (Difco), 5 % glucose, 3 % calcium carbonate and 2·5 % agar. After growth the bacteriological purity of the suspensions was checked by the following four methods: (1) microscopic appearance of the cells; (2) Gram negativity; (3) inability to grow on 0·5 % peptone-0·25 % yeast extract; and (4) the typical oxygen uptake in the Warburg apparatus with selected substrates as mentioned previously (Schell, 1960; Joubert, Bayens & De Ley, 1961).

Enzyme preparations. The cells were disrupted and the soluble and particulate fractions prepared as previously described (De Ley & Schell, 1959).

Enzyme activity. The conventional Warburg apparatus was used as previously described, as were the methods for the determination of NAD- and NADP-linked

dehydrogenases, for the detection of pyruvate decarboxylase and of the cytochromes, and for the preparation of CO and mixtures of N_2/O_2 (De Ley & Schell, 1959).

RESULTS

Comparative enzymology of lactate catabolism

An identical mechanism of lactate catabolism was found in all the strains investigated. As this system was nearly the same as the one already described for *Acetobacter aceti* (*peroxydans*) (De Ley & Schell, 1959) it may suffice to summarize the results briefly.

Oxidations by resting cells

With all the strains of the suboxydans group of Frateur (1950) 1 mole O_2/mole lactate, 1 mole O_2/mole ethanol and 0·5 mole O_2/mole pyruvate or acetaldehyde was consumed. With all the other strains the above substrates were almost completely oxidized to CO_2 and H_2O. With most of the strains D-lactate was oxidized faster than L-lactate. Addition of 10^{-2} M-arsenite resulted in a complete inhibition of the oxygen uptake beyond the acetate stage. Direct observation with a Beck–Hartridge reversion spectroscope showed that all the above substrates reduced the cytochromes.

Enzymes in the particulate fraction

One mole O_2/mole of either D- or L-lactate, 1 mole O_2/mole ethanol, 0·5 mole O_2/mole pyruvate or acetaldehyde was taken up. The pH optimum was 5–6. D-lactate was again oxidized faster than the L-isomer. Addition of cofactors (NAD, NADP, FAD, FMN or riboflavin) or artificial carriers (methylene blue or *N*-methyl phenazinium methosulphate) was not required or did not stimulate the oxidation. Oxygen uptake was inhibited 75 % by mixtures of 90 % CO and 10 % O_2 and completely by 10^{-3} M-KCN. $NADH_2$ was readily oxidized but acetate was not oxidized.

Soluble enzymes

In the absence of an artificial carrier no oxygen was taken up. In the presence of methylene blue or N-methyl phenazinium methosulphate, the same overall oxidations took place as with the particulate fraction. D-Lactate was again oxidized faster than the L-isomer. Addition of cofactors did not stimulate the reaction. In the presence of either M/640 *p*-benzoquinone, 0·1 M-dimedon or $3{\cdot}10^{-2}$ M-$NaHSO_3$, the reaction stopped after an oxygen uptake of 0·5 mole/mole lactate indicating the formation of pyruvate or acetaldehyde. Neither an NAD- nor an NADP-linked dehydrogenase for either substrate could be detected.

Mechanism of pyruvate catabolism

Here only the results obtained with strain aceti Ch 31 are described, since essentially the same situation was encountered in all the other strains. As the activity of the 'pyruvate oxidase system' depends on the substrate on which the cells have been grown, the results reported in this section were all obtained with cells grown on a glycerol medium as described under 'Methods'.

Pyruvate decarboxylase

In all the strains the soluble fraction effected a strong direct decarboxylation of pyruvate, which was completely inhibited by M/640 *p*-benzoquinone and stimulated by either 8 mg. dimedon/ml. or $3 \cdot 10^{-2}$ M-$NaHSO_3$, indicating the formation of acetaldehyde as reaction product. After removal of the cofactors by dialysis against 0·6 % versene, addition of Mg^{++} and ThPP was required for the decarboxylation (Fig. 1).

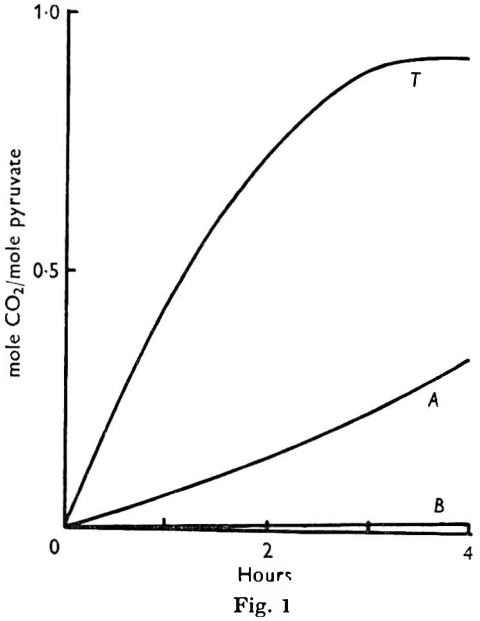


Fig. 1

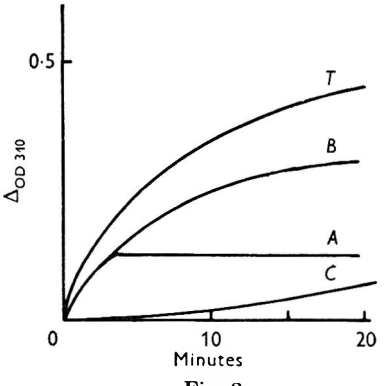


Fig. 2

Fig. 1. Requirement for thiamine pyrophosphate and Mg^{++} of the soluble pyruvate decarboxylase of *Acetobacter aceti* (*rancens*). The soluble enzyme preparation had been dialysed against 0·6 % versene in 0·02 M-phosphate buffer pH 7·4, overnight at 4°. CO_2 production was measured in the conventional Warburg apparatus at 30° in N_2 atmosphere. Curve T, 1 ml. soluble enzyme, 5 μmole $MgCl_2$, 500 μg. ThPP and 10 μmoles sodium pyruvate. Curve *A*, Mg^{++} absent. Curve *B*, ThPP absent.

Fig. 2. The oxidation of acetaldehyde with NAD by crude extract of glycerol-grown *Acetobacter aceti*, strain Ch 31. Curve *T*, 1 ml. crude extract, 200 μmoles tris buffer pH 9, 10 μmoles KCN, 0·4 μmole CoA, 5 μmoles $MgCl_2$, 10 μmoles glutathione, 0·3 μmole NAD and 10 μmoles acetaldehyde. Curve *A*, 1 ml. crude extract, 200 μmoles tris buffer pH 9, 10 μmoles acetaldehyde. Curve *B*, same as curve *A* but 10 μmoles KCN added. Curve *C*, same as curve *B*, but 10^{-2} M-arsenite added. Final volume, always 3 ml. The formation of $NADH_2$ was measured in the Beckman spectrophotometer, model DU and expressed as the increase in O.D. at 340 mμ.

Acetaldehyde dehydrogenases

Both crude extract and soluble fraction contained an NAD-linked acetaldehyde dehydrogenase. This enzyme is stimulated by CoA or glutathione and completely inhibited by 10^{-2} M-arsenite (Fig. 2). KCN and the alkaline pH inhibited the strong $NaDH_2$-oxidase. The effect of CoA and glutathione became even more pronounced after dialysis. The soluble enzyme preparation was dialysed overnight

against a suspension of Dowex 50 (H^+). The stimulation by CoA and glutathione is illustrated in Fig. 3. A very active NADP-linked acetaldehyde dehydrogenase was also present (Fig. 4). From the negligible activation by KCN and the complete reduction of NADP, it was concluded that no $NADPH_2$-oxidase was present. The NADP- and NAD-linked acetaldehyde dehydrogenases were different enzymes, since the latter was completely inhibited by 10^{-2} M-arsenite, whereas the former was not (Fig. 5*a*, *b*). Dimedon did not inhibit the reaction with NADP, whereas

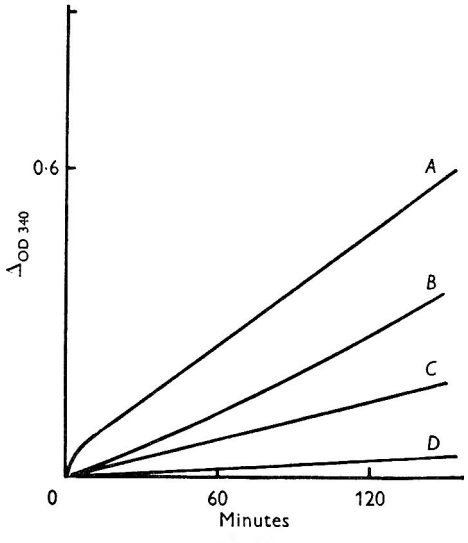


Fig. 3

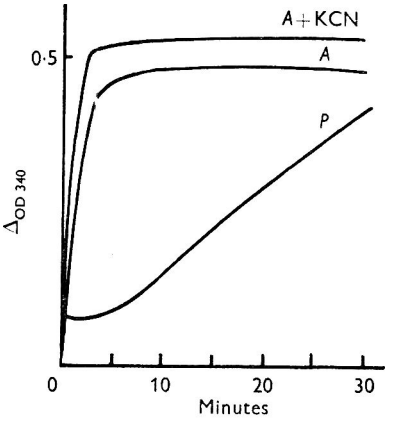


Fig. 4

Fig. 3. Effect of CoA and glutathione on the oxidation of acetaldehyde by NAD with a dialysed preparation of soluble enzymes of glycerol-grown *Acetobacter aceti*, strain Ch 31. Same conditions as in Fig. 2, but without KCN. Curve *A*, complete system. Curve *B*, CoA absent. Curve *C*, glutathione absent. Curve *D*, both CoA and glutathione absent. The results are expressed as in Fig. 2.

Fig. 4. Oxidation of acetaldehyde and pyruvate in the presence of NADP and crude extracts of *Acetobacter aceti*, strain Ch 31. Curve *A*, 1 ml. crude extract, 10 μmoles acetaldehyde, 0·3 μmoles NADP; final volume 3 ml. M/45 phosphate buffer pH 7. Curve *A* + KCN, same as for curve *A*, but 10 μmoles KCN added. Curve *P*, same as curve *A* but pyruvate instead of acetaldehyde. The results are expressed as in Fig. 2.

a marked inhibition was observed with NAD. This might be because the NADP-linked enzyme was very active, resulting in a rapid formation of $NADH_2$, before acetaldehyde reacted with dimedon. In the case of the less active NAD-linked dehydrogenase, there might be a competition between the enzyme and dimedon for acetaldehyde. The enzyme itself was not inhibited by dimedon, as seen from the initial reaction rates.

The 'pyruvate oxidase system'

According to King *et al.* (1956), cells of *Acetobacter pasteurianus*, grown on a medium with 2% glycerol, contained a pyruvic oxidase system, requiring CoA, glutathione, ThPP and NAD. We fully confirmed this observation with extracts of

glycerol-grown cells (Fig. 6). KCN and the alkaline pH inhibited the strong $NADH_2$-oxidase. Although pyruvic decarboxylase itself was partially inhibited at this pH, the results showed that the reaction was activated by CoA and GSH. They seemed to indicate the existence of a pyruvate oxidase system. On the other hand an alternative explanation might as well be that the reduction of NAD with pyruvate was not due to a pyruvate oxidase system, but to the joint action of pyruvate decarboxylase and the NAD-linked acetaldehyde dehydrogenase. The experiments shown in Fig. 7 supported this view. At higher NAD concentrations acetaldehyde was dehydrogenated at once, whereas pyruvate was oxidized after a marked induction

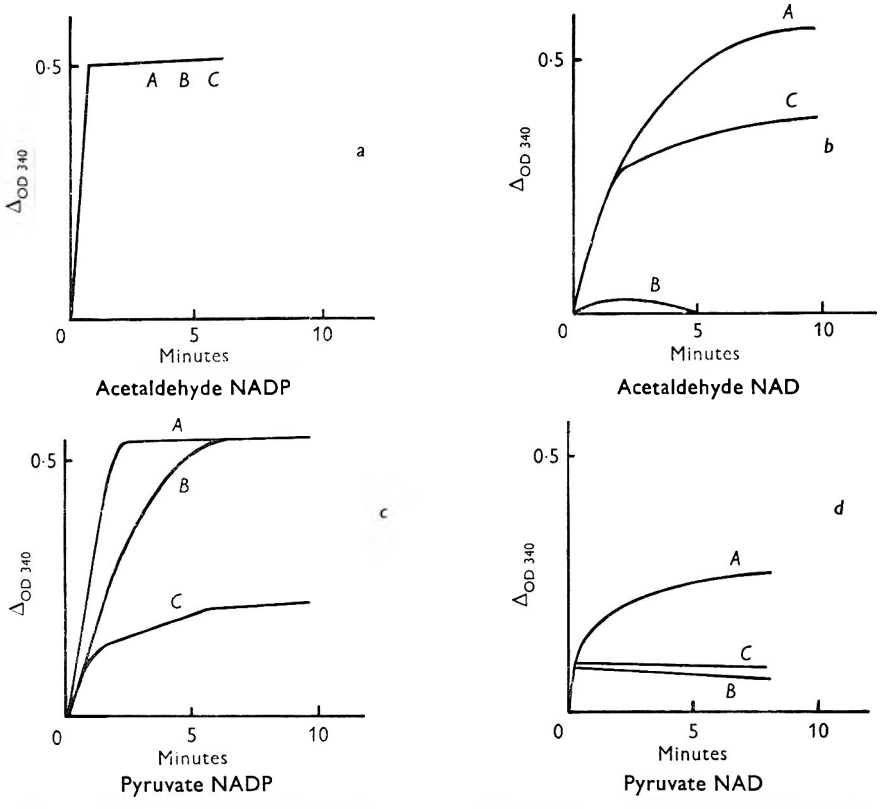


Fig. 5. Effect of dimedon and arsenite on the oxidation of acetaldehyde and pyruvate by NADP and NAD with soluble enzymes of glycerol-grown *Acetobacter aceti*, strain Ch 31. *a* and *b*, acetaldehyde as substrate. *c* and *d*, pyruvate as substrate. Curve *A*, no inhibitors. Curve *B*, 10^{-2} M-arsenite as inhibitor. Curve *C*, 8 mg./ml. dimedon as inhibitor. With NAD the basic systems contained 1·5 ml. enzyme preparation, 200 μmoles tris buffer pH 8·2, 0·3 μmoles NAD, 10 μmoles of either acetaldehyde or pyruvate. Final volume 3 ml. With NADP, 1·5 ml. enzyme preparation, M/45 phosphate buffer pH 7·0, 0·3 μmole NADP, 10 μmoles substrate in 3 ml.

period. Addition of 10 μmoles of $MgCl_2$ resulted in an appreciable shortening of the induction period. Other evidence is illustrated in Fig. 5. Dimedon inhibited NAD reduction by pyruvate almost completely and $NADPH_2$ formation quite markedly. Arsenite again hardly affected $NADPH_2$ formation and blocked $NADH_2$ formation. The results of Fig. 5 and 7 indicated that acetaldehyde was an intermediate in the

pyruvate oxidation and that a pyruvic oxidase system was not involved. It seemed possible that acetyl-CoA was involved according to the following pathway:

$$\text{pyruvate} \xrightarrow[\text{Mg}^{++}\ \text{ThPP}]{\text{CO}_2} \text{CH}_3\text{CHO} \xrightarrow[\text{NAD}]{\text{CoA, GSH}} \text{acetyl-CoA} + \text{NADH}_2.$$

Therefore the formation of acetyl-CoA was tested by the method of Lipmann & Tuttle (1945) in the system described by Kitos, King & Cheldelin (1957). However, acetyl-CoA was not detected, even when the experiments were repeated on several occasions with several modifications.

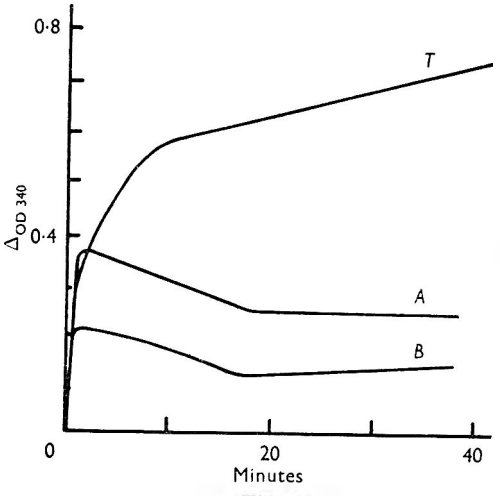


Fig. 6

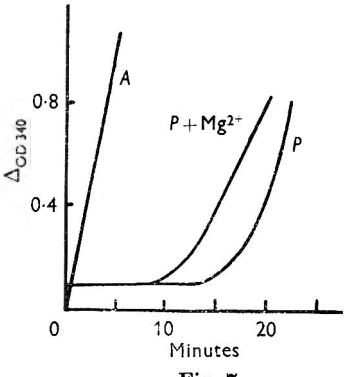


Fig. 7

Fig. 6. Apparent presence of a pyruvic oxidase system in extracts of *Acetobacter aceti*, strain Ch 31. Mixture: 1 ml. of crude extract, 200 μmoles tris buffer pH 9, 10 μmoles KCN, 0·4 μmoles CoA, 5 μmoles $MgCl_2$, 10 μmoles glutathione, 0·3 μmole NAD, 500 μg. ThPP and 10 μmoles sodium pyruvate. Final volume 3 ml. Curve *T*, complete system. Curve *A*, CoA absent. Curve *B*, both CoA and glutathione absent. The results are expressed as in Fig. 2.

Fig. 7. Oxidation of acetaldehyde and pyruvate in the presence of high NAD concentrations with crude extracts of *Acetobacter aceti*, strain Ch 31. Curve *A*, 0·6 ml. extract, 3 μmoles NAD, 10 μmoles acetaldehyde, 200 μmoles tris buffer pH 9. Final volume 3 ml. Curve *P*, same system but pyruvate instead of acetaldehyde. Curve $P + Mg^{++}$ same system as for curve *P*, but 10 μmoles $MgCl_2$ added. The results are expressed as in Fig. 2.

It is noticeable that the enzyme system for the formation of $NADH_2$ from pyruvate is dependent on the substrate on which the cells have been grown. With extracts from cells, grown on the lactate medium, no formation of $NADH_2$ could be observed, not even in a complex system containing 50 μg. CoA, 500 μg. ThPP, 10 μmoles GSH, 5 μmoles $MgCl_2$, 5 μmoles $MnCl_2$, 10 μg. lipoic acid, 10 μmoles Na_2HPO_4, 0·3 μmole NAD, 10 μmoles pyruvate, 0·3 ml. crude extract or soluble enzyme and 200 μmoles tris buffer pH 8·0.

Localization of the pyruvate decarboxylase

We shall now show that the localization of the pyruvate decarboxylase depends on the carbon source on which the cells had been grown and that the particulate enzyme is not bound in the same way as are the oxidase systems.

Resting cells of the strains aceti Ch 31, rancens 23 and xylinum 25 oxidized several substrates in a different way, depending whether they had been grown on either lactate or glycerol (Fig. 8). With lactate-grown cells, ethanol, D-lactate and acetate were oxidized completely to CO_2 and water. In the presence of 10^{-2} M-arsenite only 1 mole O_2 per mole ethanol or D-lactate was taken up, while acetate was not oxidized at all (Fig. 8*a*). With cells grown on glycerol (Fig. 8*b*) the result

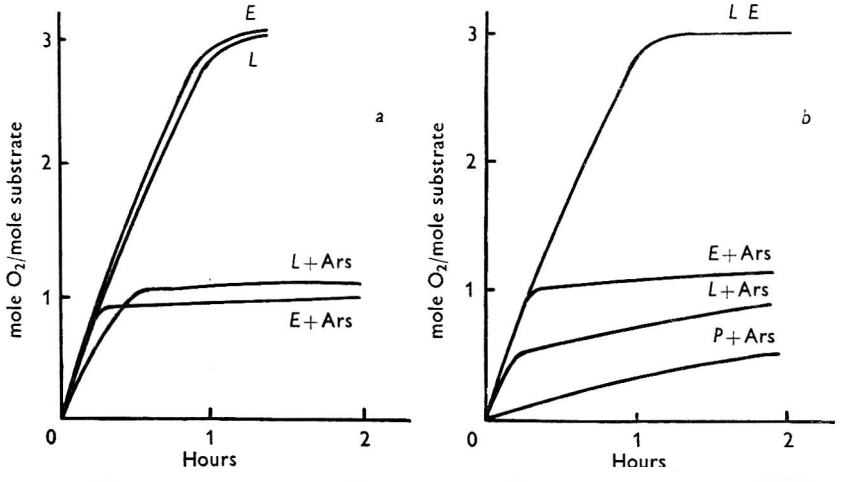


Fig. 8. Effect of 10^{-2} M-arsenite on the oxidation of ethanol and sodium D-lactate by resting cells of *Acetobacter aceti*, strain Ch 31. *a*, Cells grown on DL-lactate. *b*, Cells grown on glycerol. The oxygen uptake was measured in the conventional Warburg apparatus at 30°. Each vessel contained 80 mg. of living bacteria (wet weight) in a final volume of 3 ml. Side arm 10 μmoles substrate. 0·1 ml. 20% KOH in central well. Curves *E*, ethanol. Curves *L*, sodium D-lactate. Curves *L* + Ars, D-lactate and arsenite. Curves *E* + Ars, ethanol and arsenite. Curve *P* + Ars, pyruvate and arsenite.

was the same except for the oxidation of D-lactate in the presence of arsenite, where only 0·5 mole O_2 was taken up. Presumably either pyruvate or acetaldehyde had been formed. Pyruvate itself was not oxidized under these conditions. No pyruvate decarboxylase activity could be demonstrated in the particulate fraction prepared from the strains aceti Ch 31, rancens 23, pasteurianus 11 and suboxydans 26, grown on either glycerol or ethanol, while particles from lactate-grown cells of the strains peroxydans 8618, aceti Ch 31, rancens 23 and xylinum 25 contained pyruvate decarboxylase activity. The particulate fractions always contained active systems oxidizing lactate, ethanol and acetaldehyde. These facts might be explained if one accepts that the pyruvate decarboxylase was associated with the cytoplasmic membrane only when the cells are grown on a substrate in whose catabolism pyruvate is an intermediate e.g. lactate. When grown on another substrate, e.g. glycerol or ethanol, the decarboxylase would be present only in the cytoplasm. The observation

by Stouthamer (1960), that particles from glucose-grown *Acetobacter aceti* (*liquefaciens*) displayed no pyruvate decarboxylase activity, fits in with this hypothesis.

The particulate pyruvate decarboxylase displayed another peculiarity, in that it could easily be dislodged from the particles by simple washing with buffer. The particles were separated from the crude extract by centrifugation in the Spinco Centrifuge model L at 100,000 *g* for 2 hr. The supernatant was carefully decanted and the tube wiped dry. The gel-like material was suspended in M/50 phosphate buffer pH 6·5. An aliquot was used for the estimation of the decarboxylase activity

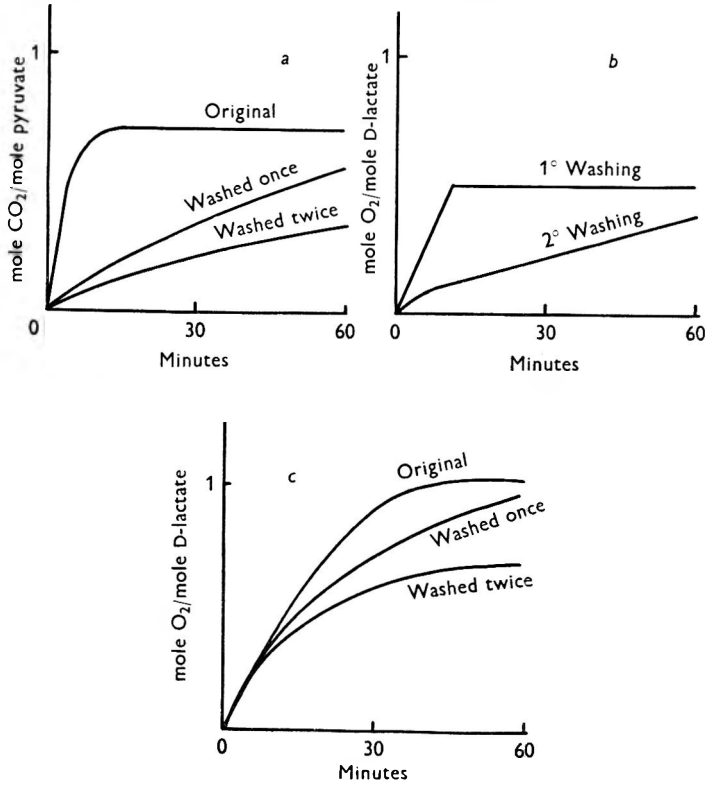


Fig. 9. Release of pyruvate decarboxylase from the particulate fraction by washing with buffer. *a*, Pyruvate decarboxylase activity in the particulate fraction of disrupted *A. aceti*, strain Ch 31, before washing and after first and second washing with M/50 phosphate buffer pH 6·5. *b*, Decarboxylase activity in the supernatant after washing and centrifugation. Enzyme activity was determined in the conventional Warburg apparatus at 30° in N_2 atmosphere. *c*, Release of D-lactate oxidase system from the particulate fraction in the same conditions as in *a*. Enzyme activity was determined in the Warburg respirometer.

(Fig. 9*a*). The remainder was again centrifuged at 100,000 *g* for 2 hr. The pellet was taken up in the same buffer and the activity redetermined. About 75 % had been lost. A third treatment resulted in a further 10 % loss of activity. That no cofactor had been washed out but the entire enzyme itself could be shown because the supernatant now contained the activity (Fig. 9*b*). This experiment in itself was of course not a proof that the enzyme was dislodged from the particles, as it could very well have been a mere washing away of the enzyme from the soluble

fraction, which was undoubtedly still trapped between the mass of centrifuged particles in the first pellet. That this was not the case was shown by the study of the behaviour of a typical soluble enzyme during the washing procedure: the lactate dehydrogenase. For activity measurements the latter enzyme was coupled to *N*-methyl phenazinium methosulphate. In the first washings already there was hardly any activity, while the second time there was no activity whatsoever. This showed that the soluble pyruvate decarboxylase, which was trapped between the particles after the first centrifugation, was negligible as compared to the amount of enzyme released during washing. In contrast to pyruvate decarboxylase, the other particulate enzymes were hardly dislodged. This is illustrated for the D-lactate oxidizing enzyme system (Fig. 9*c*), where the initial rate of oxidation was hardly affected by washing. In this case the final oxygen uptake decreased from 1 to 0·5 mole O_2/mole substrate, because the pyruvate decarboxylase had been washed off.

DISCUSSION

Mechanism and localization of lactate catabolism as an argument for phylogenetic unity in the acetic acid bacteria

The enzymic mechanism for the breakdown of lactate is essentially identical in all the strains of *Acetobacter* and *Gluconobacter* investigated. This extends our previous results obtained with *A. aceti* (*peroxydans*) (De Ley & Schell, 1959) and shows that the latter were only one example of a general phenomenon. In acetic acid bacteria both isomers of lactate and ethanol are metabolized to acetate by two sets of enzymes, which carry out the same overall reactions and pass by the same intermediates, but are of a different nature and localization. There is first the particulate system which oxidizes both D- and L-lactate by way of pyruvate and acetaldehyde to acetate and oxidizes ethanol by way of acetaldehyde likewise to acetic acid. From previous experiments (De Ley & Dochy, 1960*a*, 1960*b*) it is known that the enzymically active particles are fragments of the cytoplasmic membrane which were called 'oxidosomes' (De Ley, 1960). The membrane also contains most of the cytochromes, which serve as the electron-accepting system in the oxidations. The nature and the coenzymes of the particulate dehydrogenases for lactate, ethanol and acetaldehyde (as well as for many other substrates upon which we reported on other occasions) remain unknown. It may be remembered that neither NAD, NADP, FMN, FAD, riboflavin, nor methylene blue, phenazine methosulphate and 2,6-dichlorophenol indophenol ever showed any stimulation with the particulate fraction.

The soluble enzymes in the cytoplasm have been extensively discussed in the above paper, which need not be repeated here. From the present investigations it follows that there are only a few quantitative differences between the strains which are of minor importance such as (1) the ratio between the rates of oxidation of D- and L-lactate is not the same for all the strains, although D-lactate is oxidized always at least as fast as L-lactate; (2) the inhibition by H_2O_2 of the oxygen uptake by the oxidosomes is more pronounced in *A. aceti* (*peroxydans*) than in the other strains, but this is due to the very active peroxidases in peroxydans (De Ley & Vervloet, 1961) and the absence of catalase in this strain. It thus appears that the enzymic mechanism for the breakdown of lactate and ethanol to acetate is a common

and basic feature of all the acetic acid bacteria, since the same system with the same distribution in the cell is found in such widely divergent strains as *A. aceti* (*peroxydans*) and *Gluconobacter oxydans* (*suboxydans*). It is an important argument in favour of the phylogenetic unity of these bacteria as previously postulated (De Ley, 1961).

Mechanism of pyruvate catabolism

A survey of the literature had revealed that three different mechanisms of pyruvate breakdown appeared to be operative in acetic acid bacteria. This raised a promising question of comparative biochemistry, but it proved to be deceiving. Indeed, a pyruvate decarboxylase was found in all strains investigated. At a first glance there was also a pyruvic oxidase, since we could easily repeat the experiments of King *et al.* (1956), in which reduction of NAD by pyruvate required CoA, glutathione and ThPP. However, upon closer inspection, a different explanation becomes apparent. We have indeed shown that the same extracts contain an NAD-linked acetaldehyde dehydrogenase which is activated by CoA and glutathione and completely inhibited by 10^{-2} M-arsenite. Furthermore, the inhibition by dimedon shows that pyruvate reduces both NAD and NADP only *after* decarboxylation to acetaldehyde.

The hypothesis of a separate pyruvate oxidase system is unnecessary as the reduction of the nicotinamide nucleotides can be explained by the joint action of pyruvic decarboxylase (requiring ThPP) and an NADP- or NAD-linked acetaldehyde dehydrogenase (the last being activated by CoA and glutathione and being inhibited by arsenite). It is worth noticing that the apoenzymes for both coenzymes are different, since the NADP-linked enzyme is not inhibited by arsenite and not activated by CoA and glutathione. In addition, the NAD-linked enzyme seems to be semi-inducible, since no or doubtful activity was found in lactate-grown cells. Neither could we find evidence for the contention of Rao (1955, 1957) that *Acetobacter aceti* contains an arsenite- and dimedon-insensitive pyruvate catabolic pathway, which does not include pyruvate decarboxylase. We invariably found the latter enzyme and the influence of both inhibitors has been mentioned above. Closer inspection of Rao's results reveals that his system was apparently arsenite insensitive because glutathione was added for the preparation of the cell extracts and dimedon indeed inhibited pyruvate oxidation 33 %. The overall conclusion is thus that a pyruvate decarboxylase could be demonstrated convincingly in all the strains investigated.

It may be noted that resting cells appear to metabolize acetaldehyde by way of the NAD-linked enzyme and not by the more active NADP-linked one, as can be seen from the inhibition of the lactate oxidation by arsenite.

The localization of the pyruvate decarboxylase

All strains of acetic acid bacteria investigated so far contain pyruvate decarboxylase in the cytoplasm. Whether the cytoplasmic membrane will bear a decarboxylase depends on the substrate on which the cells are growing. Only the particles from lactate-grown cells displayed pyruvate decarboxylase activity. On the other hand, particles from cells grown on glycerol, ethanol or glucose never showed any activity of the latter enzyme. It seems that, when the enzyme plays an active role during

the growth of the bacteria, it is associated with the cytoplasmic membrane. The physiological role of this 'inducible' localization can be seen from experiments with resting cells. When the pyruvate decarboxylase is associated with the cytoplasmic membrane, lactate is oxidized to acetate completely by way of the membrane-linked system. In the other case lactate is metabolized by the membrane only as far as pyruvate, which then enters the cytoplasm and is broken down by the soluble decarboxylase. This is illustrated in Fig. 10.

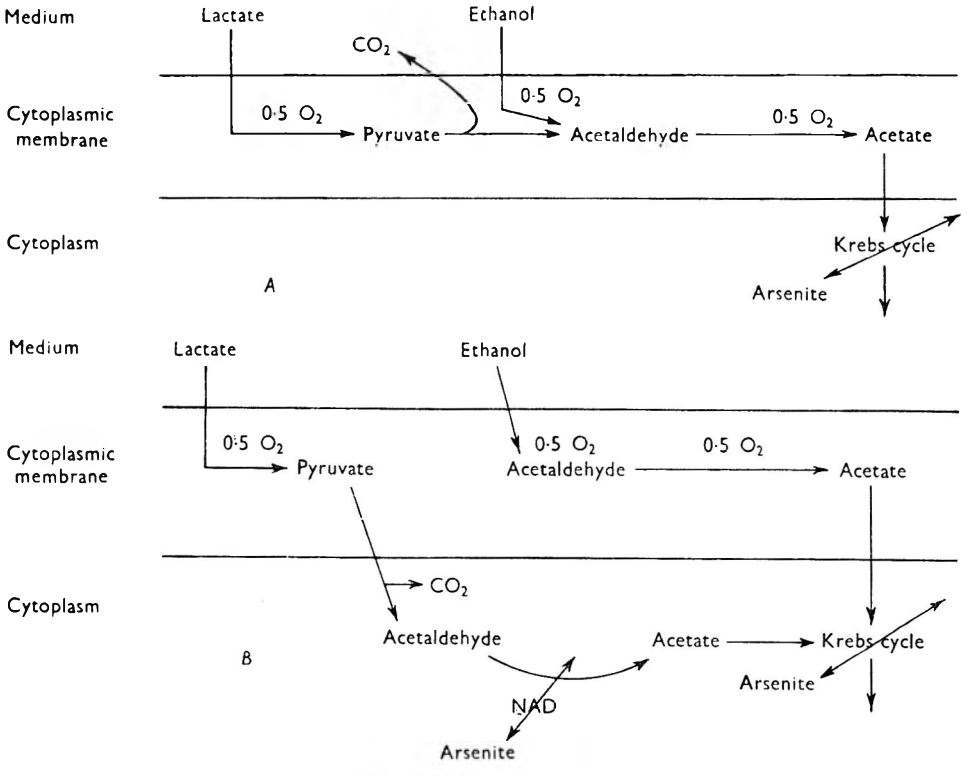


Fig. 10. Localization of the main pathway of lactate metabolism in acetic acid bacteria, grown on different substrates. *A*, cells grown on lactate; *B*, cells grown on either glycerol, ethanol or glucose.

It seems thus to be of importance for the cells that their substrates are metabolized as much as possible by a chain of enzymes which are located closely together.

The particulate pyruvate decarboxylase demonstrates a second peculiarity residing in its mode of linkage. It could indeed be washed off the particles by repeated treatment with buffer. We have not yet encountered a similar behaviour with other particulate enzymes of these bacteria, which are always tightly bound. Nothing is known of the mode of linkage of pyruvate decarboxylase to the particulate material, neither can it be stated whether the particulate enzyme is the same as the soluble one (with an inducible localization) or if they are different ones (with an inducible particulate enzyme).

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Further Observations on the Properties of Megacin, a Bacteriocin Formed by *Bacillus megaterium*

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SUMMARY

A sensitive method for the assay of mμg. amounts of megacin is described. Sensitive organisms adsorb megacin and the saturation concentration is similar to bactericidal concentrations. The death of megacin-treated organisms is, however, not coincident with adsorption but can be delayed or prevented by incubation at low temperature. Post-adsorption death is in fact proportional to the incubation temperature. Bactericidal amounts of megacin transform protoplasts of sensitive organisms to empty spherical ghosts and also appear to disrupt the permeability barrier of whole organisms. Megacin does not, however, degrade isolated or intact cytoplasmic membranes.

INTRODUCTION

Iványovics and his co-workers (Iványovics & Alföldi, 1954, 1955; Iványovics & Nagy, 1958) discovered that large numbers of strains of *Bacillus megaterium* isolated from natural sources produced specific antibacterial agents, to which they gave the name megacins. Megacins are regarded as members of the group of highly specific bactericidal substances first reported by Gratia (1925, 1932) and now known as bacteriocins. The formation of bacteriocins appears to be a lethal process (Ozeki, Stocker & de Margerie, 1959) and in particular in the case of megacin is reminiscent of the production and liberation of induced lysogenic phage (Iványovics & Alföldi, 1955, 1957; Iványovics, Alföldi & Nagy, 1959*a*). Nevertheless the bacteriocins appear to be proteins free from nucleic acids (Fredericq, 1957). The megacin produced by *B. megaterium* strain 216 is the only bacteriocin to have been purified (Holland, 1961); it is an acidic (apparently simple) protein of molecular weight 51,000. The present paper is concerned with the antigenic properties of megacin 216 and its mode of action. The development of a sensitive assay procedure for the detection of mμg. amounts of megacin has enabled the potent antibacterial activity of purified megacin to be examined quantitatively.

METHODS

Bacillus megaterium strain 216 (a megacin producer) and strain 207M (used for megacin assay) were described previously (Holland, 1961). *Escherichia coli* K 112 was obtained from Dr F. Jacob (Pasteur Institute, Paris). Peptone broth was used to grow strain 207 M, it contained (g.): 1, NaCl; 1, Oxoid peptone; 0·1, Lab. Lemco; water to 100 ml.; final pH 7·2. Glucose (0·2 % w/v, final concentration) was added

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aseptically when required. Nutrient broth contained (g.): 1, oxoid tryptone; 0·5, Difco yeast extract; 0·5, Lab. Lemco; in 100 ml. of distilled water; final pH 7·2. The preparation of other media, purified megacin and the estimation of protein all followed the methods already described (Holland, 1961).

Total organism counts were made with a Thoma haemocytometer cell; bacterial suspensions diluted to E 0·3–0·4; each count completed in triplicate.

Viable counts. The suspensions of organisms were diluted at room temperature with nutrient broth or peptone broth, to 2×10^3–4×10^3 organisms/ml.; 0·1 ml. volumes were spread on the surface of nutrient agar plates, previously warmed to 37°. The plates were incubated at 37° for 14–16 hr. and the colonies then counted. The diluted suspensions were plated in triplicate; where possible duplicate dilution series were also prepared and plated.

Plating efficiency. This is expressed as percentage viable count compared with total cell count. Well aerated cultures of *Bacillus megaterium* strain 207 M in the stationary or late logarithmic growth phase consist mostly of single cell organisms with a small proportion of two-celled organisms. When the latter were considered as single viable units, good agreement was found between total and viable counts.

Protoplasts. Protoplasts of *Bacillus megaterium* strain 207 M were prepared in phosphate buffer or nutrient broth as described by Weibull (1953) and McQuillen (1956) using 14 hr. nutrient broth cultures grown at 37° and with lysozyme (Armour Laboratories) at 100 μg./mg. dry wt. organism; the stabilizing agent was 0·2 M-sucrose. The behaviour and morphology of the resulting protoplasts resembled those described by Weibull (1953).

Cytoplasmic membrane fractions. These were obtained from protoplast preparations by the method of Weibull & Bergström (1958) in the additional presence of 10^{-2} M-NaCl + 5×10^{-3} M-$MgCl_2$. The product, a pink pellet constituting about 17 % (w/v) of the initial dry weight organism, was suspended in phosphate buffer or nutrient broth and was used immediately.

Cell wall prepararions of Bacillus megaterium *strain 207 M.* Cell walls were prepared according to Salton & Horne (1951) and suspended in a small volume of phosphate buffer or nutrient broth and kept at 4°; total recovery was 10 % (w/v) of the initial cell dry weight.

Megacin antiserum. Freshly diluted solutions of purified megacin in sterile phosphate buffer were injected intravenously twice weekly into rabbits (96 μg. protein/ml./injection). Where required the megacin was mixed (1 + 1) with an adjuvant which consisted of 9 vol. Bayol F (supplied by Esso, Queen Anne's Gate, London, S.W. 1) and 1 vol. Arlasel A (Honeywell & Atlas, Chadwell Heath, Essex). Samples of blood were taken 7 days after the completion of each series of injections; the blood was allowed to clot and the serum obtained by centrifugation.

Phosphate buffer, $Na_2HPO_4 + NaH_2PO_4$, was prepared according to Heppel (1957) and unless otherwise stated was 0·02 M and at pH 7·1.

The turbidity (E) of growing cultures or suspensions of bacteria was measured at 610 mμ with an SP 600 Unicam spectrophotometer with 1 cm. glass cells, or with an Eel nephelometer using a barium sulphate standard.

Specific activity of all preparations was defined as units megacin activity/mg. protein, megacin activity being determined by the plate assay (Holland, 1961). Samples of the purified megacin were kept in phosphate buffer at −15° and since

the activity of the preparations declined slowly over several months the specific activity of megacin was always determined immediately before use when examining the mode of action.

Bactericidal action of purified megacin under standard conditions. In addition to the assay system described previously a method was devised to detect much smaller amounts of megacin. Strain 207 M of *Bacillus megaterium* was grown in peptone broth + 0·2 % (w/v) glucose, with shaking in a water bath at 30°. The organisms were harvested at the end of the first growth phase (see Fig. 2), washed and resuspended in peptone broth + glucose to a concentration equiv. 340 μg. dry wt. organisms/ml. (E 0·72; about $1{\cdot}7 \times 10^8$ organisms/ml.). After equilibration at 37° for 10 min. the suspension was mixed with an equal volume of megacin solution in phosphate buffer. In most experiments 10·1 mμg (final concentration) of megacin-protein/ml. was used. After 20 min. the suspension was rapidly diluted and the viable count determined (at 37°) and compared with that of the untreated control suspension. With megacin preparations of specific activity $5{\cdot}63 \times 10^5$ the death of 60–90 % of the treated organisms was usually obtained in these conditions.

RESULTS

Antigenic properties of megacin

Megacin is a potent antigen which elicits the formation of both precipitating and neutralizing antibodies in rabbit serum. In the presence of adjuvant the amount of neutralizing antibody increases to a titre of 200 after 7 weeks (12 injections) and, after further injections, in the 9th week to 600 (adjuvant antiserum, see Table 1).

Table 1. *Neutralization activity of megacin antisera*

Rabbits injected twice weekly for 9 weeks, each dose 96 μg. megacin protein/injection with an adjuvant, Bayol F + Arlasel A. Twofold serial dilutions of serum in saline (final volume 0·5 ml.) mixed with 0·22 μg. megacin protein in 0·5 ml. phosphate buffer, incubated at 37° for 2 hr. kept overnight at 4°. Residual megacin activity measured by standard plate procedure. Control solutions containing megacin but no serum were included. The neutralization titres given are the means of duplicate assays.

Rabbit no.		Period (weeks)	Neutralization titre
1	Adjuvant serum	7	200
		9	600
		14	100
2	Non-adjuvant serum	7	64
		9	32
3	Non-immune serum	9	0

The injections then ceased and the antibody titre decreases to 100 after 5 more weeks. Antiserum prepared in the absence of adjuvant (non-adjuvant antiserum) reached a maximum titre only of 64 and the sera from a non-immune animal did not inhibit megacin activity at a 1/2 dilution. The precipitin titre of different sera in presence of megacin in saline was also determined (Table 2). With antiserum (adjuvant) precipitates were formed at all concentrations of megacin tested; the maximum precipitate was obtained at a megacin concentration of 22 μg. protein/ml. The supernatant fluids from all these suspensions were not active; the precipitates

when resuspended were also not active. Thus the antiserum precipitated and neutralized the megacin antigen; non-immune serum and non-adjuvant antiserum did not precipitate or neutralize megacin under these conditions.

Table 2. *Precipitation titre of megacin antiserum*

Rabbit serum obtained as in Table 1 (7 weeks) diluted 1+1 with 0·5 ml. aliquots incubated in 4 ml. test tubes with 0·5 ml. megacin solution (prepared in saline), 2 hr. at 37° and kept at 4° for 72 hr. Precipitation scored by inspection, supernatants assayed for megacin after centrifuging.

Megacin protein final concn. (μg./ml.)	Supernatant activity (10^{-3} units megacin/ml.)			Degree of precipitation		
	Adjuvant serum	Non-adjuvant serum	Non-immune serum	Adjuvant serum	Non-adjuvant serum	Non-immune serum
88	0·50	10·0	10·0	++	—	—
44	0·05	5·0	5·0	+++	—	—
22	0·05	1·0	1·0	++++	—	—
11	0·05	1·0	—	+++	—	—
5·5	0·05	0·01	0·10	++	—	—
2·8	0·05	0·01	0·10	+	—	—
0	—	—	—	—	—	—

Antibacterial activity of megacin

Bacillus megaterium strain 207 M was grown in nutrient broth for 14 hr. and the early stationary phase organisms were harvested, washed and resuspended in broth. Samples (1 ml.) were equilibrated at 37° and mixed with 1 ml. samples of sterile phosphate buffer containing different amounts of megacin. After 20 min. each sample was diluted 2500 times in broth to prevent further adsorption of megacin; after further dilution the number of surviving colony formers was determined. The number of organisms killed was proportional to the megacin concentration (Fig. 1) except at the highest values where increasing megacin concentration caused only a small increase in the number of organisms killed. Although there was some variation from experiment to experiment a concentration of 1·5–6·0 mμg. megacin preparation/ml. was required to kill 50 % of the organisms in these conditions. In the experiments where megacin was most active, calculation showed about 100 molecules megacin/organism (Fig. 1).

The mechanism of action of megacin

Conditions for assay of megacin

To carry out a quantitative investigation of the bactericidal action of megacin, a method for the detection of minute amounts of the antibacterial agent was required; several technical difficulties had to be overcome before a reproducible system was obtained. Although the experiments described above (Fig. 1) were completed successfully, other similar experiments failed because of the premature death (often associated with lysis) of most of the untreated as well as the treated cells. The main requirements were three-fold to obtain: (1) organisms of uniform size consisting as far as possible of single cells; (2) physiologically active suspensions which when subjected to a variety of environmental conditions would still plate

with a high efficiency, and (3) organisms which gave a reproducible response to megacin under standard conditions. To meet the first of these requirements organisms were always harvested from the stationary phase since at this stage the culture consisted almost entirely of single cells. (Moreover these cells, unlike those from the exponential phase, were not so prone to autolysis.) However, the plating efficiency of suspensions of stationary phase organisms was often only 5–10 %, even when there was no visible lysis. In fact it was found, on measuring the growth of strain 207 M, that shortly after reaching the stationary phase the viable count

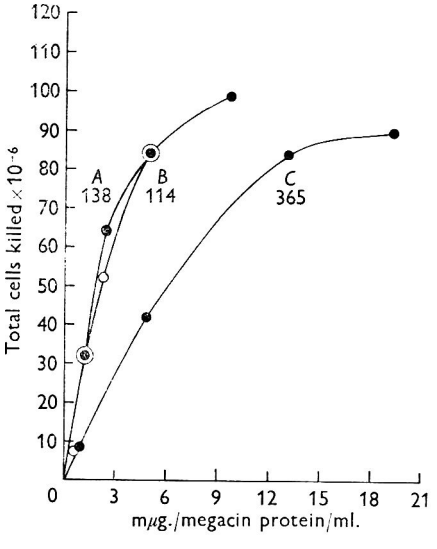

Fig. 1

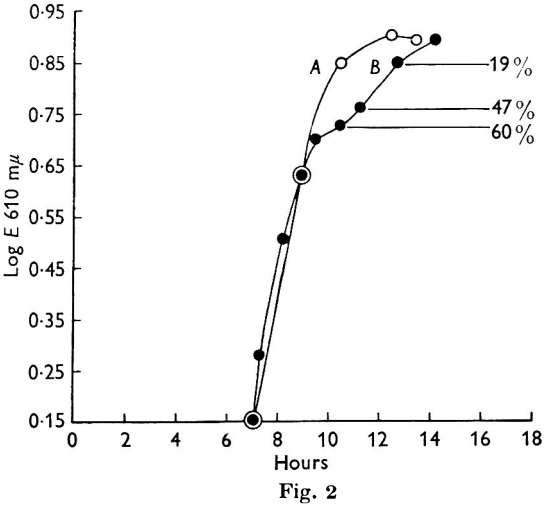

Fig. 2

Fig. 1. Effect of concentration of megacin on viable count of *Bacillus megaterium* strain 207 M after 20 min. exposure at 37°. Total kill calculated from the viable count of untreated suspensions. *A*, Total count $1{\cdot}5 \times 10^8$ organisms/ml. (plating efficiency, 57 %), specific activity megacin = $1{\cdot}60 \times 10^6$ units/mg. protein; *B*, total count $2{\cdot}3 \times 10^3$ organisms/ml. (plating efficiency, 56 %), specific activity = $1{\cdot}60 \times 10^6$ units/mg. protein; *C*, total count 2×10^8 organisms/ml., (plating efficiency, 65 %) specific activity megacin = $5{\cdot}63 \times 10^5$ units/mg. protein. Figures by the curve are LD50 values in molecules of megacin/organism.

Fig. 2. Growth of *Bacillus megaterium* strain 207 M in peptone broth + glucose at 30°, subcultured directly from nutrient broth agar slopes (curve *A*), and after several subcultures in peptone broth + glucose (curve *B*). Effect of treating organisms of increasing 'age' with megacin (10·1 mμg./ml.) at 37° for 20 min. is shown as percentage kill, indicated by the curve. This was calculated from the viable count of the control untreated suspensions. Specific activity of megacin = $5{\cdot}63 \times 10^5$ units/mg. protein.

usually decreased abruptly. In some cases the turbidity of the culture decreased, indicating that autolysis also occurred. During further studies of the growth of strain 207 M in a variety of conditions it was found that when this strain was grown at 30° in peptone broth + glucose (0·2 %), the viable count remained high in the stationary phase. It was subsequently found that the kind of growth curve obtained in this medium depended upon the source of the inoculum. When maintained on nutrient broth agar slopes, immediate subculture in liquid peptone broth + glucose gave the growth pattern shown in Fig. 2 (curve *A*). After several sub-

cultures in peptone broth + glucose however, the growth pattern was as shown in curve *B*. The cultures which showed the 2-step or diauxic growth curve could invariably be harvested between 10–14 hr. to provide washed suspensions whose viable count remained at a high value for several hours. (Plating efficiency, 60–80 %.) In contrast, suspensions obtained from single-step growth cultures were occasionally unstable; moreover the discontinuity in the growth curve of the 2-step growth cultures provided an indicator for harvesting under standard and reproducible conditions. *Bacillus megaterium* strain 207 M was therefore subsequently maintained on peptone broth + glucose agar slopes from which the first subculture showed a diauxie-like growth.

Effect of age of organisms on the response to megacin action

The instability of organisms harvested before 10 hr. (in peptone broth + glucose cultures) prohibited their use for experimental purposes; but the effect of megacin on suspensions of organisms after this was examined. The sensitivity of the bacteria decreased markedly (after 10 hr.) (Fig. 2, curve *B*). Examination of wet mounts of organisms, in the presence of Indian ink (Duguid, 1951) showed that this decrease in sensitivity was concurrent with the progressive development of an extensive slime layer (Pl. 1). It is reasonable to suppose that the slime layer prevented or retarded the penetration of megacin to those structures where it exerted its lethal activity. There is, however, no evidence to indicate that this is a major or a specific mechanism of resistance to megacin.

Adsorption of megacin by Bacillus megaterium *strain 207 M*

In the subsequent experiments organisms were harvested after 10 hr. growth in peptone broth + glucose and treated with 10·1 mμg. megacin-protein/ml. (see Methods). This procedure usually resulted in the death of 60–90 % of the bacteria and combined the use of maximum yield of viable organisms harvested under relatively standard conditions with a maximum sensitivity of these organisms to megacin. Adsorption of megacin by sensitive bacteria was now examined. Suspensions of *Bacillus megaterium* strain 207 M, strain 216 and *Escherichia coli* K 112 were prepared and exposed to megacin at 37° in polythene centrifuge tubes (previously sterilized by ultraviolet irradiation); after 20 min. the organisms were quickly centrifuged down and discarded. Control tubes containing megacin but no bacteria were taken through the same procedure. Megacin remaining in the supernatant fluid was estimated by mixing with equal volumes of fresh suspensions of strain 207 M and determining the number of surviving organisms after exposure to the antibiotic for 20 min. The results of three experiments are summarized in Fig. 3. In each case megacin appeared to be completely adsorbed by the 207 M bacteria; the adsorption by the other strains was variable. In all cases the degree of adsorption by the megacin-sensitive strain was higher than by the other two strains.

Saturation level of adsorption by Bacillus megaterium *strain 207 M*

To verify that megacin adsorption by strain 207 M was specific, the saturation concentration for megacin uptake was determined and compared with those concentrations known to be bactericidal. Suspensions of sensitive bacteria were mixed

with different concentrations of megacin; after 20 min. at 37° the suspensions were centrifuged and the bacteria discarded. Two controls containing no bacteria but 22·1 and 10·1 mμg. megacin/ml. respectively, were treated similarly. The supernatant fractions were used to test fresh suspensions of strain 207 M, the viable count of which was determined after 20 min. at 37°. The results are shown in Fig. 4. Bacteria mixed with 44·4 mμg. megacin protein/ml. did not appear to take up all the protein since some megacin activity was detected in the supernatant fluid. At greater concentrations progressively larger amounts of megacin were unadsorbed. A concentration of rather less than 44 mμg. protein/ml. was therefore sufficient to saturate all the adsorption sites. Incubation with the megacin controls (final concentration, 11·1 and 5·0 mμg. megacin protein/ml.) caused the death of 31 % and 14 % of the viable bacteria (Fig. 4). The concentration of megacin that is necessary to saturate the adsorption sites is therefore within the range of the bactericidal concentration of megacin.

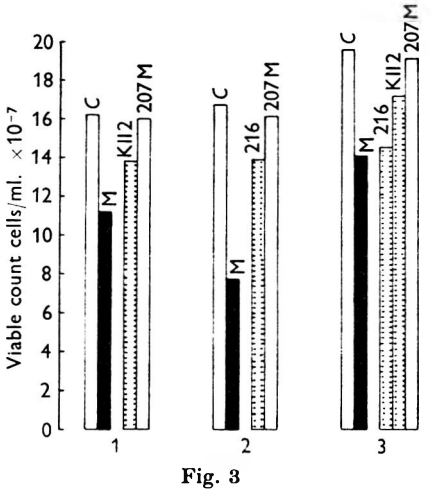

Fig. 3

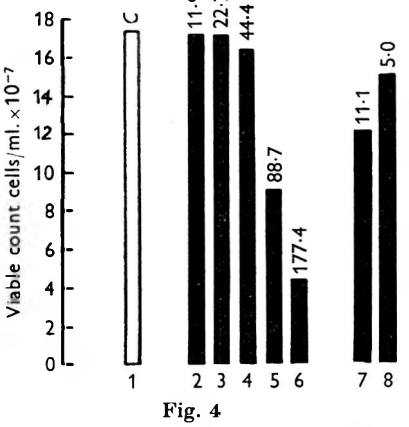

Fig. 4

Fig. 3. Adsorption of megacin by bacteria. Histograms show viable count of *Bacillus megaterium* strain 207 M, after each treatment. Controls (*C*, open blocks) no megacin; solid blocks, 20 min. with 5·0 mμg. megacin/ml.; remaining blocks show survival of *Bacillus megaterium* strain 207 M after exposure to the supernatants obtained after preliminary adsorption of megacin, (10·1 mμg./ml.) by suspensions of *Escherichia coli* (K 112), *B. megaterium* 216 (216) and *B. megaterium* 207 M (207 M). Specific activity megacin = 5·63 units/mg. protein.

Fig. 4. Residual megacin activity after adsorption by *Bacillus megaterium* strain 207 M. Suspensions of cells mixed with various concentrations of megacin at 37°, centrifuged after 20 min. Fresh suspensions of *B. megaterium* 207 M added to supernatants and viable organisms counted after 20 min. Open block, no megacin; solid blocks with initial megacin concentration as stated. Blocks 7 and 8, viable counts following direct exposure to solutions of known megacin content. Specific activity megacin = $5{\cdot}63 \times 10^5$ units/mg. protein.

Survival of megacin-treated organisms at low temperature

Although adsorption was essential to its bactericidal action, at least one more step is required before the effect of megacin becomes irreversible. Suspensions of

Bacillus megaterium 207 M were incubated with megacin at 37° for 20 min., diluted and plated on groups of plates. These were incubated at different temperatures and the colonies counted (Fig. 5). The viable count of the untreated suspension was independent of the final incubation temperature but after megacin treatment it was inversely proportional to the incubation temperature. Thus, although initially exposed to megacin in identical conditions, 31 % of the organisms survived when incubated at 37° whereas 80 % survived if incubated at 15°. The Q_{10} (27°–37°) for loss of viability under these conditions was close to 2 (Fig. 6) indicating the possible involvement of an enzymic system.

Adsorption of megacin and death of the organism are therefore two distinct processes since organisms can grow at low temperature, despite the adsorption of

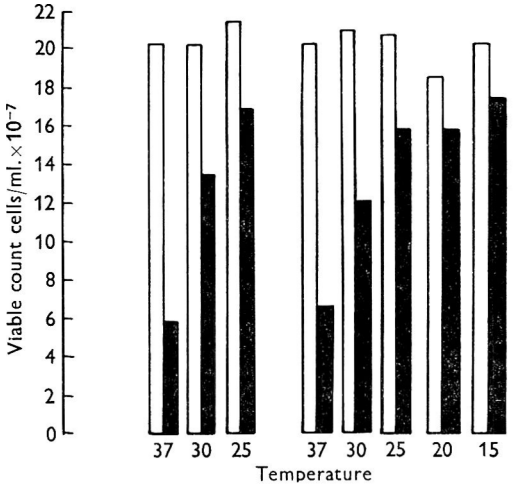

Fig. 5

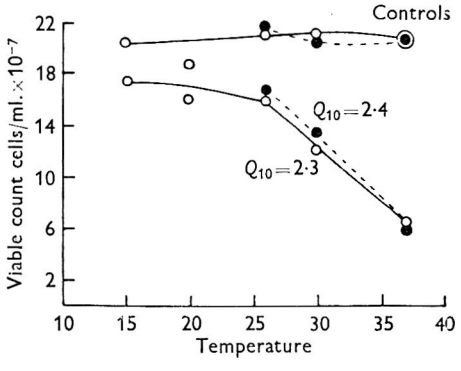

Fig. 6

Fig. 5. Effect of temperature on viability of *Bacillus megaterium* strain 207 M after adsorption of megacin at 37°. Organism treated with 10·1 mμg. megacin/ml., diluted, plated and incubated at different temperatures. Open blocks, untreated controls; solid blocks, surviving cells at different temperatures.

Fig. 6. Effect of temperature on viability of *Bacillus megaterium* strain 207 M after adsorption of megacin at 37°. Organisms treated with 10·1 mμg. megacin/ml., diluted, plated and incubated at different temperatures. Two experiments are shown, specific activity of megacin in each case = $5{\cdot}63 \times 10^5$ units/mg. protein.

Table 3. *Effect of post adsorption temperature on the lethal effect of megacin*

Bacillus megaterium strain 207 M was harvested after 10 hr. and resuspended in broth (E. 0·71). The suspensions mixed with megacin (10·1 mμg./ml. at 37° for 20 min.) before diluting and plating. Treated and non-treated cells kept at 20° for 2 hr. or 4 hr. before incubating at 37° for 14 hr.

		Time zero		2 hr. at 20°		4 hr. at 20°	
Expt.		Controls	Megacin	Controls	Megacin	Controls	Megacin
1	Viable count	$2{\cdot}01 \times 10^8$	$1{\cdot}46 \times 10^8$	$2{\cdot}13 \times 10^8$	$1{\cdot}67 \times 10^8$	$1{\cdot}93 \times 10^8$	$1{\cdot}76 \times 10^8$
	Cells killed	—	25 %	—	22 %	—	9 %
2	Viable count	$1{\cdot}82 \times 10^8$	$7{\cdot}80 \times 10^7$	$2{\cdot}32 \times 10^8$	$1{\cdot}32 \times 10^8$	—	—
	Cells killed	—	57 %	—	43 %	—	—

a potentially lethal amount of megacin. Recovery, however, is a slow process, treated organisms requiring an appreciable time at 20° before they can form colonies at 37°, e.g. 4 hr. at 20° enabled 64 % of affected organisms to grow at 37° (Table 3). After 2 hr. at 20° up to 24 % of affected organisms survived. Treated organisms kept at 4° did not subsequently recover, confirming the enzymic nature of recovery.

Action of megacin on cell permeability

Working with partially purified preparations, Ivánovics, Alföldi & Nagy (1959*b*) concluded that the megacin produced by *Bacillus megaterium* strain 216 disrupted the osmotic barrier of sensitive organisms. Purified megacin acts in the same way

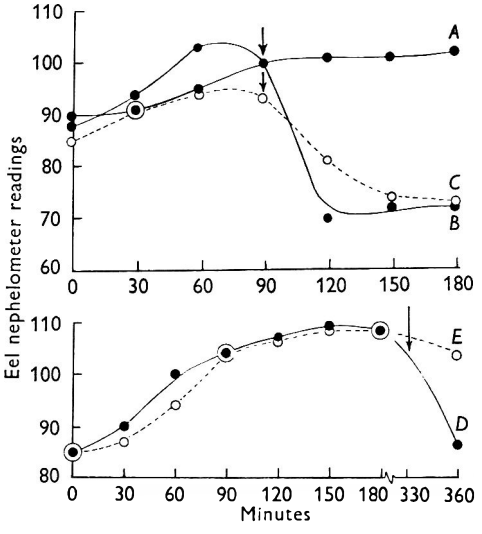

Fig. 7. Action of megacin on protoplasts of *Bacillus megaterium* strain 207 M. Nutrient broth suspensions of protoplasts (9·8 ml.) in side arm flasks equilibrated at 37° for 60 min. Megacin (0·2 ml.) in phosphate buffer added at time zero, changes followed in an Eel nephelometer. *A*, Control no megacin; *B*, 962 mμg. megacin/ml.; *C*, 481 mμg. megacin/ml.; *D*, 48 mμg. megacin/ml.; *E*, 4·8 mμg. megacin/ml. Arrows indicate appearance of protoplast ghosts observed in the phase contrast microscope. Specific activity megacin = $5{\cdot}63 \times 10^5$ units/mg. protein.

converting protoplasts of *Bacillus megaterium* strain 207 M prepared in broth, to empty spherical ghosts (Fig. 7); this occurred abruptly after a lag period (e.g. 90 min. with the highest megacin concentration). Coincident with the appearance of ghosts, turbidity of the suspension declined. The minimum concentration of megacin producing ghosts was 48 mμg. megacin protein/ml., and was comparable with that required to kill 60–80 % of treated organisms under similar conditions. Whole organisms treated with megacin at 37° and examined under the phase contrast microscope showed a gradual loss of intracellular material; after 3–6 hr. incubation a few organisms were less refractile and large granules previously almost obscured by the cytoplasm became clearly visible. By 18 hr. the suspension was almost clear and the majority of organisms contained only numerous large granules usually

concentrated at the periphery. The cell envelope, however, appeared to remain intact. In contrast, suspensions of untreated organisms did not show a significant decline in turbidity even after 18 hr. and individual organisms appeared normal.

DISCUSSION

By using a homogeneous purified preparation of megacin at a minimum bactericidal concentration effects due to an excess of the agent have been avoided, and side reactions due to unsuspected trace contaminants minimized. Similarly, by carrying out the experiments with stable cell suspensions (plating efficiency 60–80 %) megacin action was measured in relatively quantitative terms. Purified megacin, like the partially purified preparations obtained by Iványovics *et al.* (1959*b*) appeared to destroy the permeability barrier of sensitive organisms and protoplasts.

Concentrations producing these changes were similar to those killing such cells, which suggests that the disruption of the permeability barrier and protoplast membrane is a primary cause of the lethal action of megacin. The conclusions of Iványovics *et al.* (1959*b*) that megacin does not appear to attack the cell wall of sensitive bacteria is supported by the work described above; furthermore, cell wall preparations of *B. megaterium* 207 M in broth, did not decrease in turbidity when incubated at 37° with the antibiotic (74 mμg. megacin/mg. dry weight cell walls). More interesting, however, was the failure of megacin under the same conditions to alter the turbidity of protoplast membrane fractions of sensitive bacteria, while the ghosts formed by megacin action or protoplasts retained their original size and shape. Hence megacin does not destroy the macrostructure of the protoplast membrane in the intact protoplast or in isolated preparations, and other reasons must be sought for the permeability changes which it induces. If megacin were an esterase like lipase one would expect the protoplast to be degraded, with visible changes in turbidity (cf. Spiegelman, Aronson & Fitz-James, 1958). If megacin acts like a lysozyme similar changes would be expected with cell wall preparations (cf. Salton, 1957). Iványovics *et al.* (1959*b*) found evidence of neither proteolytic nor detergent activity in their megacin preparations. The very low concentration at which megacin is active (1–20 mμg./ml.) contrasts sharply with the bactericidal concentration of typical surface-active agents, e.g. with polymixin and gramicidin a concentration of 1–25 μg./ml. is required to produce an effect (Newton, 1956; Erlanger & Goode, 1954). That such low concentrations of megacin could have any direct osmotic effect on sensitive cells is unlikely. Megacin it seems brings about a subtle change within the osmotic membrane and whilst such changes may be caused by a highly specific enzyme, there is as yet no direct evidence for this.

Saturation of the adsorption sites of sensitive organisms requires very low concentrations of megacin which undoubtedly explains the failure of Iványovics *et al.* (1959*b*) to demonstrate adsorption, since they tested for uptake by the relatively insensitive plate assay. This adsorption process is not necessarily lethal, since despite having adsorbed a potentially lethal dose of megacin, organisms may recover and remain viable if placed in a suitable environment, e.g. temperatures of the order of 20°. It seems probable that the lesion produced by megacin is concomitant with adsorption but that it is not necessarily fatal unless the organisms

are immediately exposed to optimum growth conditions. So far experiments with growing organisms have been prohibited by the instability of *Bacillus megaterium* strain 207 M when subjected to environmental changes. Unfortunately no comparison with the mode of action of other bacteriocins is yet possible since little data, especially with purified compounds, is available.

I wish to record my most grateful thanks to Dr B. A. Fry for his continued interest and helpful discussion of this work and for his invaluable help in the preparation of the manuscript. I have greatly appreciated the interest taken by Professor S. R. Elsden and the help and advice given by all members of this Department. I am also very grateful to Dr J. K. A. Beverly for supplying the megacin anti-serum. Finally I wish to express my thanks to the Medical Research Council for a Training Scholarship.

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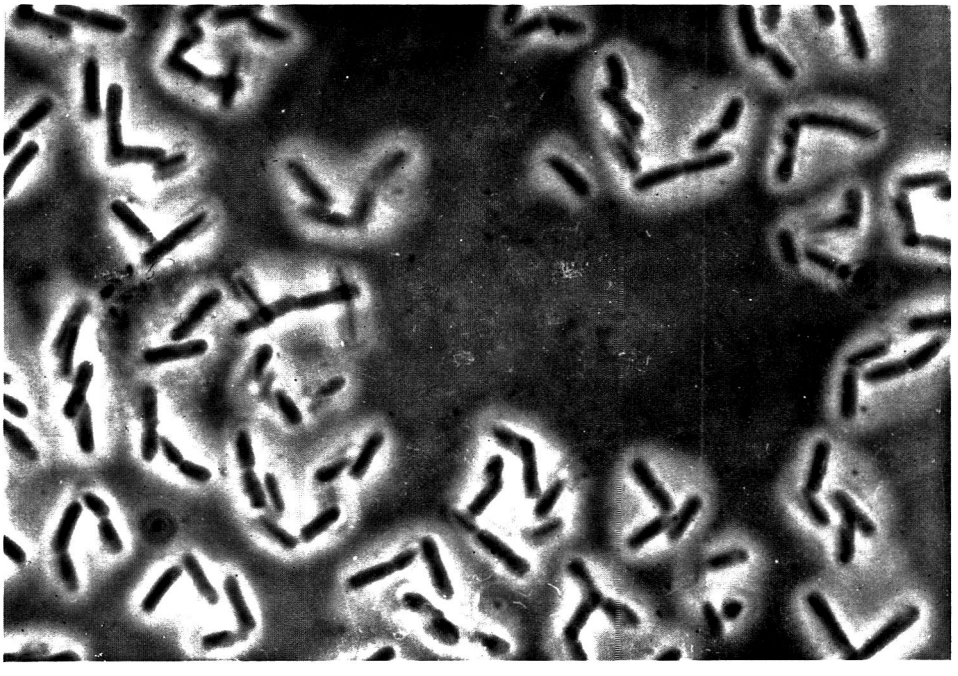
EXPLANATION OF PLATE 1

Bacillus megaterium strain 207 M grown 10 hr. in peptone glucose broth showing development of slime layer. Wet mounts of washed cells prepared according to Duguid (1951) × 1500.



I. B. HOLLAND

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With 1 plate
Printed in Great Britain

Simonsiellaceae fam.nov. with Characterization of *Simonsiella crassa* and *Alysiella filiformis*

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Department of Microbiology, University of Queensland, Brisbane, Australia

(*Received* 18 *April* 1962)

SUMMARY

The cultural, biochemical and morphological characteristics of six cultures of *Simonsiella* and four cultures of *Alysiella* are described. The multicellular filaments of both *Simonsiella* and *Alysiella*, originally termed 'disk-bacteria', are ribbon-like, non-sporing, non-branching, aerobic and Gram-negative. They are greater than 2 μ wide, exhibit gliding motility on solid media and ferment carbohydrates. The family Simonsiellaceae fam.nov. is proposed to include the genus *Simonsiella* Schmid and the genus *Alysiella* Langeron. Amended descriptions of the species *Simonsiella crassa* Schmid and of *Alysiella filiformis* (Schmid) Langeron are given.

INTRODUCTION

In 1906 Müller discovered the 'Scheibenbakterien' or 'disk-bacteria' in tooth sediment and saliva of man and in throat mucus of a chicken (Simons, 1922); Müller (1911) was not sure of their classification as bacteria. Simons (1922) rediscovered these micro-organisms and also examined one of Müller's original preparations whereupon he recognized them as members of the Cyanophyceae. Most of the later workers agreed with Simons who regarded them as Oscillatoria-like forms which had become colourless by adaptation to parasitic or saprophytic conditions. Simons described three species that were classified in the genus *Simonsiella* by Schmid (Simons, 1922). The type species, *S. muelleri*, occurred in the oral cavity of man and various domestic animals; *S. crassa*, found in the same habitat, was distinguished by its greater width; *S. filiformis* was present in the mouth mucus of domestic animals but, because it differed morphologically, Simons questioned its inclusion in the genus *Simonsiella*. He regarded them all as harmless saprophytes. Langeron (1923) excluded *S. filiformis* from the genus *Simonsiella* and placed it in the new genus *Alysiella*. To illustrate the basis of generic differentiation Langeron defined both genera. The following is a free translation:

Genus *Simonsiella*: Colourless, non-motile trichomes formed of short elements; from 7–20 μ long and 2–3 μ wide without cap-cells or apical thickenings but with a mucilagenous envelope; divided by numerous transverse septa into narrow compartments whose width does not exceed 0·4–0·7 μ; numerous hormogonia 3–5 μ long arising from constriction of the trichomes; cytoplasm very basophilic, rarely granular; spores unknown.

Genus *Alysiella*: Colourless, non-motile trichomes of variable and indefinite length; flat, striped, segmented into articulated units like segments of a tapeworm;

units are all alike; no mucilagenous envelope; very small hormogonia formed from one or more units generally paired; cytoplasm basophilic, often granular; spores unknown.

METHODS

Unless stated otherwise cultures were incubated aerobically at 37°. Liquid media were dispensed in 5 ml. quantities in 150 × 13 mm. test tubes. When first isolated the cultures required 10 % serum (horse or ox) added to Oxoid nutrient agar. Therefore 10 % serum was included in the various test media except those used for nutritional studies. Selected cultures known to give positive reactions and uninoculated controls, all containing serum, were included.

Source and isolation of cultures. Borax methylene blue-stained smears of rabbit and sheep saliva revealed the organisms by their characteristic morphology. Oral swabs were plated out on 10 % serum agar and incubated at 37° for about 6 hr., by which time microcolonies, mainly over deposited epithelial cells, were visible by phase-contrast microscopy. These were transferred to new media with a micro-instrument designed by Professor V. B. D. Skerman (to be published).

Several pure cultures were obtained from rabbits and from sheep. Those from rabbits were difficult to maintain and are being reserved for subsequent research. Ten cultures (A1 to A4 and S1 to S6) from sheep were used for this study. These represented two distinct morphological forms.

Preservation of cultures. Cultures were grown at 37° in 10 % horse serum in Oxoid nutrient broth (final pH 7·4) and were subcultured every 7–10 days. Cultures were freeze-dried by using 1–2 day serum agar slope cultures washed with culture-drying fluid. This fluid was prepared by mixing 3 volumes of sterile serum with 1 volume of a Seitz-filtered solution containing 12 % peptone and 30 % glucose.

Staining and morphology

Heat-fixed smears prepared from 6-hr. cultures on serum agar were stained by the Gram procedure, by 0·1 % basic fuchsin (1 min.) and by 3 % acid fuchsin (1 min.). Representative cultures S6 and A1 were stained for flagella by the method of Leifson (1951, 1958).

General morphology and motility were observed by phase-contrast studies of microcolonies on agar plates and slide cultures. A clean sterile glass slide placed on a flat agar base in a Petri dish was covered with a thin layer of serum agar. When set the serum agar was inoculated with several drops of a young serum broth culture distributed uniformly over the slide area. After incubation for about 6 hr. the slide was cut out. Examination was made by using the low power phase-contrast objective for evidence of slime trails before applying a coverglass and observing with the oil immersion phase-contrast objective for morphology and any evidence of cell movement. Cultures were also examined for motility in liquid medium by a hanging drop procedure (Skerman, 1959).

Photomicrographs were taken on Adox KB14 film with a Leica 35 mm. camera attached to a Reichert phase-contrast RC microscope.

Metabolism and nutrition

Anaerobic growth. Serum agar plate cultures were incubated anaerobically for 4 days by using the pyrogallol plate method (Skerman, 1959) and the McIntosh & Fildes jar.

Growth temperature. Serum broth cultures were incubated in water baths at temperatures of 20°, 37°, 45° and 60° for 24 hr.

pH *Value for growth.* Serum broths adjusted to pH values of 4, 5, 6, 7, 8, 9 and 10 were inoculated and examined for growth after incubation for 7 days. Phenol red indicator was included in the medium to detect local pH reaction in control tubes during incubation.

Utilization of citrate as the sole source of carbon. The Oxoid product of Simmons citrate agar medium (Oxoid Manual, 1961, 2nd ed.) was inoculated and incubated for 4 days.

Growth requirements. Plates of serum agar, Oxoid nutrient agar, and peptone yeast extract (PYE) agar were inoculated and incubated for 2 days, then examined for growth. PYE agar was prepared by dissolving 1 % (w/v) Bactopeptone (Difco), 0·5 % (w/v) Bacto yeast extract (Difco), 0·5 % (w/v) NaCl, and 1·4 % (w/v) Bacto agar (Difco) in distilled water, adjusting to pH 7·2 and then autoclaving at 121° for 20 min.

Fermentation of carbohydrates

Preliminary tests with 5 ml. of carbohydrate serum water in a 150 × 13 mm. tube did not show any acid production from glucose. The test was positive, however, in a liquid medium prepared by adding 1 % carbohydrate and 0·1 % phenol red to serum broth dispensed in 0·5 ml. quantities in 75 × 13 mm. flat-bottomed test tubes. A moist atmosphere during incubation was required to prevent evaporation. Similar reactions were obtained with a solid medium consisting of 1 % carbohydrate and 0·1 % phenol red in serum agar poured as a layer over a nutrient agar base. Both media were inoculated with drops of serum broth cultures.

Biochemical tests

Hydrolysis of aesculin. The medium described by Sneath (1956) was modified by adding 10 % serum and altering to pH 7·4. After incubation for 4 days the plates were examined for presence of the brown colour indicating aesculin hydrolysis.

Hydrolysis of casein. Oxoid nutrient agar containing 20 % skim milk was poured in 5 ml. quantities over a saline agar base. Plates were incubated for 2 days.

Litmus milk. The medium was prepared as described by Skerman (1959) with spray-dried powdered milk; incubation period 7 days.

Indole production. Serum broth + 1 % tryptone was incubated for 3 days then tested for indole by adding Ehrlich–Böhme reagent.

Methyl red (M.R.) *and Voges-Proskauer* (V.P.) *tests.* Serum (10 %) was added to the glucose phosphate peptone water medium (Kauffmann, 1954) and dispensed in 4 ml. quantities. The tests were performed after incubation for 4 days by the methods recommended by Skerman (1959); O'Meara's modification of the V.P. test was used.

Production of H_2S. Serum broth containing 0·01 % cystine and 0·05 % sodium

sulphate was used. Production of H_2S was detected by the lead acetate paper method recommended by Skerman (1959); incubation period 7 days.

Liquefaction of Loeffler's inspissated serum. Sterile horse serum was added to the sterile glucose peptone water as recommended by Skerman (1959) and 15 ml. quantities inspissated at 80° for 30 min. in Petri dishes. Plates were incubated for 4 days.

Haemolysis. Oxoid nutrient agar containing 10 % citrated horse blood was inoculated by the pour-plate method. Plates were examined after incubation for 18 hr.

Methylene blue reduction. One drop of 1 % aqueous methylene blue was added to each 24 hr. serum broth culture. The result was observed after re-incubation for 30 min.

Reduction of nitrate to nitrite. Peptone water and serum broth media containing 0·2 % KNO_3 were inoculated and tested according to the procedure of Skerman (1959).

Catalase. The test described by Sneath (1956) was performed on 1–2 day serum agar cultures.

Urease. The medium recommended by Skerman (1959) was modified by adding 10 % serum and including only 1 % urea, since 2 % urea in serum agar inhibited growth of the cultures. The incubation period was 7 days.

Hydrolysis of gelatin. The medium recommended by Skerman (1959) was prepared with Oxoid nutrient agar as the base. Plates were incubated for 4 days then tested with acid mercuric chloride.

Hydrolysis of starch. PYE agar was the basal medium used to prepare starch agar described by Skerman (1959). After incubation for 4 days hydrolysis was tested for by flooding the plates with 96 % (v/v) ethanol in water and examining for zones of clearing.

Glucose utilization. Hugh & Leifson's medium (1953) containing 1 % glucose as carbohydrate was modified by adding 10 % serum. Incubation period was 2 weeks. A second method, described by Lysenko (1961), was also tested. This medium was modified by adding 10 % serum and adjusting to pH 7·4, then dispensed in 0·5 ml. quantities in 75 × 13 mm. flat-bottomed test tubes. The reaction was observed after incubation for 1 week in a moist atmosphere.

Hippurate hydrolysis. The test was performed as described by Ayers & Rupp (1922) but was modified by adding 10 % serum to the medium.

RESULTS

Staining reactions and morphology

All cultures stained Gram-negative and were basophilic. There was no evidence of flagella. Examination by phase-contrast microscopy presented the following morphological features.

Strains S 1 *to* S 6. These organisms (see Pl. 1, figs. 1–4) were multicellular filaments consisting of a series of closely apposed cells, about 0·6 μ long and 3–4 μ wide, with the terminal cells hemispherical (compare Oscillatoria). They appeared to divide by constriction into hormogonia-like units that occasionally remained attached for some time. Each unit was 4·5 μ long. Multi-unit filaments 54 μ or longer have been observed.

Unlike Oscillatoria the multicellular unit was flat, not cylindrical, and measured 1–1·5 μ in thickness. The organisms frequently turned on their edges and curled up (Pl. 1, fig. 2). Phase-dense areas occurred centrally in the intercalary cells and as apical thickenings in the terminal cells (Pl. 1, figs. 1, 4).

The filaments exhibited active gliding motility in young cultures when the broad base was presented to the solid medium. They showed no translational movement whilst on their edges. Pl. 1, fig. 3, shows organisms at the beginning of migration from a microcolony. Pl. 1, fig. 4, shows the positions of organisms at intervals of 10 min. Slime trails were visible by low power phase-contrast examination of unmounted colonies.

Table 1. *Fermentation of carbohydrates by Simonsiella and Alysiella strains*

	Cultures									
	S1	S2	S3	S4	S5	S6	A1	A2	A3	A4
Carbohydrate	Reactions									
Arabinose	A	A	A	A	K/A	K/A	K	K	K	K
Rhamnose	K	K	K	K	K	K	K	K	K	K
Ribose	A	A	A	A	A	A	K	SA	K	K
Sorbose	K	K	K	K	K	K	K	K	K	K
Xylose	K	K	K	K	K	K	K	K	K	K
Glucose	A	A	A	A	A	A	A	A	A	A
Fructose	A	A	A	A	A	A	A	A	A	A
Galactose	K	K	K	K	K	K	K	K	K	K
Mannose	K	K	K	K	K	K	K	K	K	K
Lactose	K	K	K	K	K	K	K	K	K	K
Sucrose	A	A	A	A	A	A	A	A	A	A
Maltose	A	A	A	A	A	A	A	A	A	A
Trehalose	A	A	A	A	A	A	A	A	A	A
Melibiose	K	K	K	K	K	K	K	K	K	K
Cellobiose	K	K	K	K	K	K	K	K	K	K
Raffinose	K	K	K	K	K	K	K	K	K	K
Melezitose	K	K	K	K	K	K	K	K	K	K
Inulin	A	A	A	A	A	A	K	SA	K	K
Glycerol	K	K	K	K	K	K	K	K	K	K
Erythritol	K	K	K	K	K	K	K	K	K	K
Mannitol	A	A	A	A	K	K/A	K	K	K	K
Sorbitol	K	K	K	K	K	K	K	K	K	K
Dulcitol	K	K	K	K	K	K	K	K	K	K
Inositol	K	K	K	K	K	K	K	K	K	K
Salicin	K	K	K	K	K	K	K	K	K	K

A, acid; SA, slight acid; K, alkaline to phenol red; K/A, inconsistent, i.e. sometimes acid, sometimes alkaline.

Strains A1 *to* A4. This morphological type is illustrated in Pl. 1, figs. 5, 6. They consisted of multicellular filaments whose cells appeared to occur in pairs with a relatively weak linkage between each pair or group of 4 cells. The filaments were of uniform width throughout and the terminal cells were similar to every other cell of the filament in being flat or slightly biconcave. Each cell was 2–3 μ wide and about 0·6 μ long. Like the preceding strains (S1 to S6) the filaments of strains A1 to A4 were flat, each cell being 0·5 μ thick. Phase-dense areas were sometimes present in the cells as swellings and usually occurred along one side of the filament

(Pl. 1, figs. 5*a*, 6*a*). Length of the filaments was variable. Gliding motility was observed in young cultures as a result of which a portion of the filament was often presented in its flattened lateral aspect (Pl. 1, figs. 5*b*, 6*b*). Low power-phase contrast examination of agar plate cultures often revealed numerous slime trails forming a network around the colony.

Cultural characteristics

None of the strains produced pigment or odour. At 24 hr. a ring of turbid growth occurred about 25 mm. below the surface of each serum broth culture, while granular and slightly turbid growth appeared in the top 36 mm. layer of liquid cultures. Pellicle formation was rare. As incubation continued the granular growth was deposited down the side and at the base of the tube until finally only sediment remained, leaving the upper liquid clear. Dispersion of the sediment produced moderate turbidity, although the granular nature often persisted, particularly in cultures A1 to A4.

Table 2. *Results of other biochemical tests with strains of Simonsiella and Alysiella*

	Cultures									
	S1	S2	S3	S4	S5	S6	A1	A2	A3	A4
Aesculin hydrolysis	−	−	−	−	−	−	−	−	−	−
Casein hydrolysis	+	+	+	+	+	+	−	−	−	−
Litmus milk	P	P	P	P	P	P	N	N	N	N
Indole	−	−	−	−	−	−	−	−	−	−
Methyl-red	−	−	−	−	−	−	−	−	−	−
Voges–Proskauer	−	−	−	−	−	−	−	−	−	−
H_2S production	+	+	+	+	+	+	+	−	+	+
Serum liquefaction	+	+	+	+	+	+	−	−	−	−
Haemolysis	*β*	*β*	*β*	*β*	*β*	*β*	*β*	*β*	*β*	*β*
Methylene blue reduction	−	−	−	−	−	−	−	−	−	−
$NO_3 \rightarrow NO_2$	−	−	−	−	+	+	−	−	−	−
Catalase	+	+	+	+	+	+	+	+	+	+
Urease	−	−	−	−	−	−	−	−	−	−
Gelatin hydrolysis	+	+	+	+	+	+	X	−	+	X
Starch hydrolysis	−	−	−	−	−	−	−	−	−	−
Glucose utilization*	F	F	F	F	F	F	F	F	F	F
Hippurate hydrolysis	−	−	−	−	−	−	−	−	−	−

+, Positive; −, negative; X, weak positive; P, peptonization; N, no change; F, fermentative.
* Results of the Hugh & Leifson test indicated fermentation of glucose by all cultures. Parallel results were obtained by Lysenko's iodoacetate method with cultures S1 to S6, but alkali production by cultures A1 to A4 obscured the reaction.

At 24 hr. the serum agar colonies of all strains were punctiform, low convex, undulate, smooth, butyrous, amorphous and translucent. As cultures aged the colonies flattened, enlarged to 1–3 mm. and often became slightly friable in consistency. Fairly rapid degeneration was indicated by decrease in phase density of the organisms in 48 hr. colonies. Colonies of strains A1 to A4 showed a tendency to spread.

Metabolism and nutrition

Anaerobic growth. No growth occurred when plates were incubated in a McIntosh & Fildes jar, but cultures S5 and S6 produced growth when the pyrogallol method of obtaining anaerobiosis was used.

Growth temperature. Only the cultures incubated at 37° produced growth.

pH *value for growth.* Cultures grew at pH 7 to 9.

Utilization of citrate as sole carbon source. No culture grew on Simmons citrate medium.

Growth requirements. Growth on nutrient agar and PYE agar was poor as compared with growth on serum agar. Cultures S1 to S6 produced better growth on nutrient agar than on PYE agar; cultures A1 to A4 showed the reverse effect.

Fermentation of carbohydrates and other biochemical tests

The results are summarized in Tables 1 and 2.

DISCUSSION

Work on Simonsiella and Alysiella spp. has been published by several authors but, to my knowledge, none since 1924. Pure cultures were apparently not studied since isolation of the organisms was not successful and the investigations consisted mainly of morphological studies of stained preparations. The morphological characteristics and habitat of cultures S1 to S6 leave no doubt that they belong to the genus *Simonsiella* Schmid, 1922. The width of the filament admits them to the species *S. crassa* Schmid, 1922. An insufficient number of strains has been studied as yet to venture an opinion on the justification for the retention of more than one species on the basis of cell width. It has, however, been observed that the width of the isolated cells tends to decrease with long serial subcultivation. Cultures A1 to A4 are so similar in morphology to the published illustration of *S. filiformis* Schmid, 1922, that there can be little doubt of their identity. The question is whether they should be classified in the genus *Simonsiella* Schmid, 1922 or in the genus *Alysiella* Langeron, 1923.

Grassé (1924) reported the presence of spores in Alysiella which he considered to be endosporous trichobacteria having affinities with Arthromitus. Endospores were not seen in any of 10 cultures examined here. Langeron and Simons did not observe spores.

Fellinger (1924) was the only author to report motility of Simonsiella. She observed slow gliding motility in a hanging-drop preparation of saliva but was unable to demonstrate organs of motility. She suggested that unilateral contraction of the segmented structure might produce this movement. Fellinger (1924) and Dannenberg (1924) reported both genera as Gram-negative.

In *Bergey's Manual* (1948) Peshkoff placed the Simonsiella species described by Simons (1922) in the genus *Caryophanon*. They were re-instated in the genus *Simonsiella* by Breed (*Bergey's Manual*, 1957).

In my opinion the described morphological and biochemical differences between strains S1 to S6 and strains A1 to A4 are sufficiently great to recognize the genus *Alysiella* Langeron, 1923, with *Alysiella filiformis* as the type species. Strain S6 (for *Simonsiella crassa*) and strain A1 (for *Alysiella filiformis*) have been lodged with the National Collection of Type Cultures, London, and are proposed as neotype cultures. They have been assigned the accession numbers NCTC 10283 and NCTC 10282 respectively.

Simonsiella was classified as a member of the family Caryophanaceae in the order Caryophanales, arranged by Peshkoff in 1940 and revised by Breed in 1955, and

appeared as such in *Bergey's Manual* (1957). However, the present investigation of Simonsiella and Alysiella has revealed certain features that exclude them from the Caryophanales, namely their peculiar flat form and their gliding motility. This latter character, combined with multicellularity, associates them more closely with the Beggiatoales. Pending the possible erection of a new order it is recommended that the two genera *Simonsiella* and *Alysiella* be reclassified into the order Beggiatoales as members of the family Simonsiellaceae *fam.nov.* of which the genus *Simonsiella* Schmid, 1922, is the type genus. The descriptions of the new family and the included genera are as follows:

Simonsiellaceae fam.nov. Multicellular organisms, strongly compressed in ribbon-like filaments. Gliding motility.

There are two genera, *Simonsiella* Schmid and *Alysiella* Langeron.

Simonsiella Schmid, 1922. Multicellular organisms, strongly compressed in ribbon-like filaments. Filaments divide into hormogonia-like units in which the individual cells are closely apposed and the terminal cells are rounded. Motile with gliding motility.

Alysiella Langeron, 1923. Multicellular organisms, strongly compressed in ribbon-like filaments. Organisms appear to occur in pairs in filaments which tend to break into groups of four or more cells in which the terminal cells are not rounded. Motile with gliding motility.

The amended descriptions of *Simonsiella crassa* Schmid and *Alysiella filiformis* (Schmid) Langeron are as follows:

Simonsiella crassa Schmid, 1922, emendavit

Multicellular, unbranched, non-sporing, ribbon-like filaments 3–4 μ wide and 1–1·5 μ thick consisting of closely apposed cells 0·6 μ long with the free faces of the terminal cells rounded. The filaments appear to divide by constriction into hormogonia-like units about 4·5 μ long which may remain attached for some time. Filaments may attain a length of 50 μ or more. Phase dense areas may occur centrally in the intercalary cells and as apical thickenings in the terminal cells. The filaments exhibit gliding motility when the broad face is presented to the solid medium but are immotile and show a pronounced tendency to curl when on their edges. Gram-negative, basophilic.

Agar colonies. After 3–4 days, low convex, 1–3 mm. in diameter, smooth, undulate, translucent. Pigment and odour absent. Broth: granular and turbid giving a moderate whitish sediment. Aesculin: no hydrolysis. Gelatin: liquefied. Casein: hydrolysed. Litmus milk: peptonized. Starch: no hydrolysis. Sodium hippurate: no hydrolysis. Inspissated serum: liquefied. Blood agar: β-haemolysis. Methylene blue not reduced. Nitrate may be reduced to nitrite. Catalase-positive. Urease-negative. Indole not produced. Methyl red test negative. Voges–Proskauer test negative. Hydrogen sulphide produced. No growth on Simmon's citrate medium. Carbohydrates are fermented (not oxidized); acid but no gas produced from glucose, fructose, ribose, sucrose, maltose, trehalose, inulin; and variable acid production from arabinose and mannitol, on agar or in shallow liquid media. Acid not produced from rhamnose, sorbose, xylose, galactose, mannose, lactose, melibiose, cellobiose, raffinose, melezitose, glycerol, erythritol, sorbitol, dulcitol, inositol or salicin.

Aerobic. Optimum temperature 37°. Source: isolated from sheep saliva.

Alysiella filiformis (Schmid, 1922) Langeron, 1923, emendavit

Multicellular, unbranched, non-sporing ribbon-like filaments whose cells appear to occur in pairs with a relatively weak linkage between each pair or group of 4 cells. The filaments are of uniform width throughout and the terminal cells are similar to every other cell of the filament in being flat or slightly biconcave. Each cell is 2–3 μ wide, about 0·6 μ long and 0·5–1 μ thick. Phase-dense areas may be present in the cells and often occur along one side of the filament. Length of filaments is extremely variable. Gliding motility is exhibited on solid media. Gram-negative, basophilic.

Agar colonies: after 3–4 days, low convex, 1–1·5 mm. in diameter, smooth, undulate, translucent and often with a narrow fringe of gliding filaments and slime trails. Pigment and odour absent. Broth: granular and turbid giving a moderate whitish sediment. Aesculin: no hydrolysis. Gelatin: liquefaction slight or absent. Casein: no hydrolysis. Litmus milk: unchanged. Starch: no hydrolysis. Sodium hippurate: no hydrolysis. Inspissated serum: no liquefaction. Blood agar: β-haemolysis. Methylene blue not reduced. Nitrate not reduced to nitrite. Catalase-positive. Urease-negative. Indole not produced. Methyl red test negative. Voges–Proskauer test negative. Hydrogen sulphide may be produced. No growth on Simmon's citrate medium. Carbohydrates are fermented (not oxidized); acid but no gas produced from glucose, fructose, sucrose, maltose and trehalose; variable acid production from ribose and inulin, on agar or in shallow liquid media. Acid not produced from arabinose, rhamnose, sorbose, xylose, galactose, mannose, lactose, melibiose, cellobiose, raffinose, melezitose, glycerol, erythritol, mannitol, sorbitol, dulcitol, inositol, or salicin.

Aerobic. Optimum temperature 37°. Source: isolated from sheep saliva.

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EXPLANATION OF PLATE 1

Young cultures on serum agar examined by phase contrast microscopy.

Fig. 1. *Simonsiella crassa.* Note the phase dense areas present centrally (*a*) within the cells and as apical thickenings (*b*) in terminal cells.

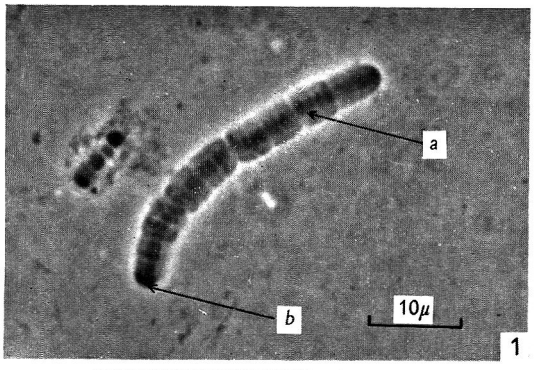
Fig. 2. Microcolony of *Simonsiella crassa* showing filaments coiled and on their edges.

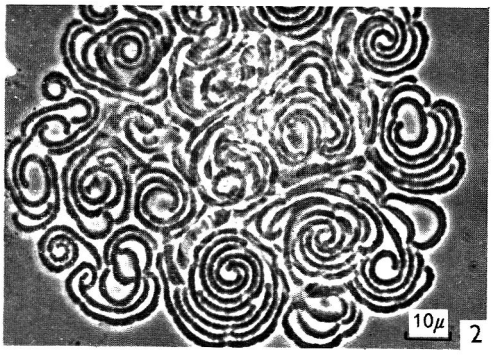
Fig. 3. Microcolony of *Simonsiella crassa* showing commencement of migration of filaments.

Fig. 4. Positions of gliding filaments of *Simonsiella crassa* at intervals of 10 min. ((i)–(iii)). Note (*a*) and (*b*) as in Fig. 1.

Fig. 5. Microcolony of *Alysiella filiformis* showing very long filaments. Note phase dense areas (*a*) and lateral view (*b*) of cells.

Fig. 6. Microcolony of *Alysiella filiformis* showing very short filaments. Note (*a*) and (*b*) as in Fig. 5.


a
b
10μ
1


10μ
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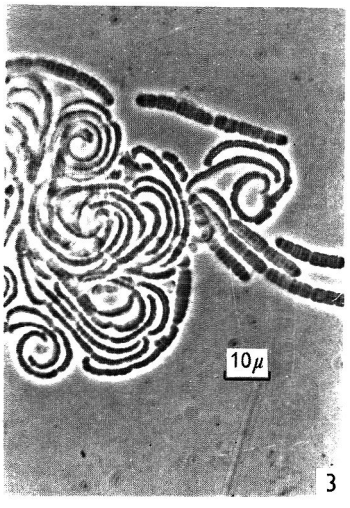
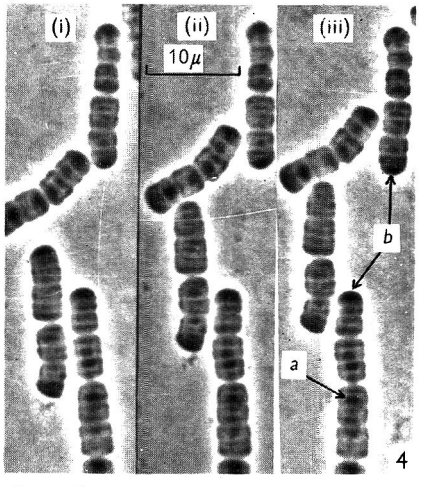
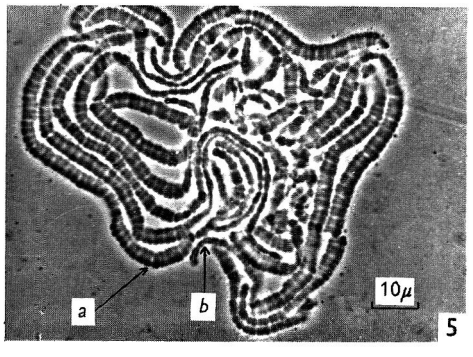
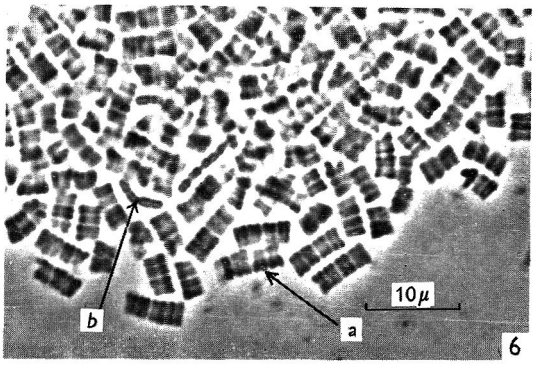

10μ
3


(i)
(ii)
10μ
(iii)
b
a
4


a
b
10μ
5


b
a
10μ
6

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The Immediate Fate of Staphylococci after Phagocytosis

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SUMMARY

Four methods were employed to study the immediate fate of staphylococci within leucocytes after phagocytosis. These were: (*a*) The method of Cohn & Morse where leucocytes and staphylococci were mixed in tubes and kept agitated. Periodically samples were removed and lightly centrifuged to sediment the leucocytes. Plate counts were made on the lysed leucocytes to measure the intracellular organisms, the supernatant to measure the extracellular population, and a non-centrifuged sample to determine the total number of viable cocci present. (*b*) Concentrated suspensions of leucocytes and staphylococci were packed together by brief centrifugation to permit rapid phagocytosis without antibodies then diluted in cold Hanks solution to stop further phagocytosis. The suspension was then rapidly passed through a Servall centrifuge with the continuous flow attachment to remove excess extracellular organisms. The infected leucocytes were placed in suspension and samples removed and treated as in the previous procedure. (*c*) Leucocytes and staphylococci were placed in plastic chambers containing a number of small coverslips. The cells were allowed to sediment and adhere to the coverslips, then washed to remove most of the extracellular organisms. Coverslips were removed at intervals and attached leucocytes lysed to liberate intracellular cocci which were enumerated by plate counts. Companion coverslips were washed, fixed, and stained in order to count the number of leucocytes present. The extracellular staphylococcal population was estimated by making plate counts on the tissue culture medium in the chambers. (*d*) Suspensions of infected leucocytes were diluted and placed in a series of Petri dishes. The cells were permitted to settle and attach to the glass. Periodically the plates were washed and melted trypticase soy agar was added. After incubation the plate counts afforded an estimation of the viable cocci remaining within the leucocytes at each sampling.

The results obtained with these procedures were in fairly good agreement with each other. It was found that *Staphylococcus aureus* (18-Z and Smith strains) survived in significant numbers within monocytes and polymorphonuclear leucocytes of normal rabbits for several hours. Little destruction of this organism during the first hour after phagocytosis could be demonstrated.

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INTRODUCTION

In previous publications (Kapral & Shayegani, 1959; Shayegani & Kapral, 1962) the authors reported that *Staphylococcus aureus* usually survived for several hours within polymorphonuclear leucocytes and monocytes of normal rabbits, but were eventually slowly destroyed within the monocytes. *S. albus*, however, was rapidly destroyed by these cells under the same conditions. While these studies were in progress, Cohn & Morse (1959) reported that *S. aureus* (Smith strain) and *S. albus* were equally rapidly destroyed within rabbit polymorphonuclear leucocytes (over 99 % within 2 hr.). Specific rabbit immune serum was used to aid phagocytosis of the organism. Using a similar method, Mackaness (1960) claimed more than 90 % destruction of the Smith strain within rabbit monocytes in 1 hr. Rogers & Melly (1960), however, indicated that *S. aureus* (Smith strain) survived in significant numbers within human polymorphs when phagocytosed in the presence of normal human serum whereas *S. albus* was destroyed. Cohn & Morse, Mackaness, and Rogers & Melly, all used the same experimental technique (originally described by Maaløe) and the same organisms, yet their results failed to agree as to the extent by which *S. aureus* was killed. Melly, Thomison & Rogers (1960) by direct observation with phase microscopy, demonstrated that 63 % of *S. aureus* (Smith strain) survived 20 min. within human polymorphs; in contrast, *S. albus* was destroyed in this interval. In the latter study all experiments were performed with blood from a single donor.

Since the conflicting results of different investigators could possibly be due to differences in experimental technique or to the use of different strains of *Staphylococcus aureus* and since our previous studies did not supply any detailed information concerning the fate of staphylococci immediately after phagocytosis, an attempt was made in the present study to apply and compare different methods of studying the fate of staphylococci during this period.

The following procedures were thus designed to study the early phase of intracellular parasitism.

METHODS

Staphylococcus aureus strains. *S. aureus* strain 18-Z; the characteristics of this strain were described previously (Kapral & Shayegani, 1959). From this strain two mutants were derived: (i) 18-Z, ad^-—an adenine-requiring mutant, which grew slowly without added adenine, and had a lag phase of about 7–10 hr.; (ii) 18-Z, SM_d—a streptomycin-dependent mutant with a moderately high frequency of back-mutation; about 1 in 60,000 can grow in the absence of streptomycin. Populations of this strain show no significant increases in streptomycin-free medium for at least 10 hr. These mutants appeared identical with the parent strain in all other *in vitro* characteristics.

Staphylococcus aureus strain Smith: was obtained through the courtesy of Drs Z. A. Cohn and S. I. Morse of the Rockefeller Institute, New York.

Leucocytes. Monocytes and polymorphonuclear leucocytes were obtained from rabbits as described previously (Kapral & Shayegani, 1959). In some experiments polymorphs were obtained by injecting 30 ml. of 0·1 % glycogen in saline intraperitoneally into rabbits a day before, and again 3 hr. before collection of the cells.

Tissue-culture procedures

Tissue-culture chamber procedure. Described in detail in Kapral & Shayegani, (1959).

Method of Cohn & Morse. As described by Cohn & Morse (1959) with the following modifications: (*a*) the tubes were incubated in a roller drum (26 cyc./min.) at 37°; (*b*) the leucocytes were disrupted with 5% sterile saponin solution; (*c*) viable leucocyte counts were also done on each sample using 1% trypan blue.

Suspended culture methods. Cells were maintained in siliconized 50 ml. Erlenmeyer flasks which had been altered so as to have a side port. The cells were kept suspended by means of Teflon-covered stirring bars rotated (30 r.p.m.) by a series of magnets connected with a belt driven by a variable speed motor. The flasks were continually gassed with 5% CO_2 in air. Sodium bicarbonate was added to the medium to give a pH of 7·2 under this atmosphere. Samples were removed at desired intervals through the side port.

Monocytes or polymorphonuclear leucocytes were induced in rabbits in the same manner already described, the peritoneal cavity was washed with 100 ml. Hanks solution containing heparin and 2% normal rabbit serum. This suspension was passed through four layers of sterile gauze and then centrifuged at 250*g* for 10 min. The sediment was resuspended in 2 ml. Hanks solution containing 10% normal rabbit serum. Leucocyte counts were made on a diluted sample of this suspension. The leucocyte suspension was mixed with a suspension of washed staphylococci (ratio of 25 cocci per leucocyte) in a conical centrifuge tube, and was packed by centrifugation at 100*g* for 3 min., to permit rapid phagocytosis without requiring antibodies. The supernate was decanted, the sediment was resuspended in 1 l. of cold Hanks solution and rapidly passed through a continuous flow centrifuge (Servall) with a two-channel distributor at 7700*g*, to eliminate most of the extracellular organisms. One litre of infected cell suspension would pass through the centrifuge in 3–4 min. The sedimented leucocytes from the two centrifuge tubes were pooled in 1 ml. Hanks solution with 10% normal rabbit serum and transferred to the culture flask containing 10–12 ml. medium (10% normal rabbit serum in Hanks solution).

After mixing, the zero-time sample was removed from the flask with a 1 ml. pipette. The entire process from the time the staphylococci were added to the leucocytes to removal of zero time samples from the flask required 18–20 min.

Samples at various intervals were treated as follows: one half ml. of sample was transferred into 4·5 ml. cold saline in a Wassermann tube, centrifuged at 50*g* for 4 min. Then 0·5 ml. of the supernatant was diluted and plated on trypticase soy agar to measure the extracellular staphylococcal population. The remaining supernatant was discarded. The sedimented cells were lysed with 1 ml. of 5% sterile saponin solution in order to release the intracellular organisms. This lysed cell preparation was also diluted and plate counts made. Two-tenth ml. of the sample was also transferred into 0·8 ml. of cold 5% saponin in another Wassermann tube, mixed, diluted, and plated out to enumerate the total number of organisms in the system. One-tenth ml. of the sample was mixed with 0·2 ml. of 1% trypan blue to count viable leucocytes.

Plate methods. The leucocytes and organisms were packed in a conical tube as

previously described and kept in a water bath at 37° for 15 min. The supernatant was decanted and the sediment washed three times with 100 ml. cold Hanks solution to remove most of the extracellular organisms. In some experiments the infected cell suspensions prepared for the suspended cultures were used.

The infected cell suspension was diluted in Hanks solution. Five ml. samples were distributed to a series of Petri dishes. The cells settled on the bottom of the dishes at 37° for 15 min. and were then washed twice with 5 ml. quantities of Hanks solution, melted trypticase soy agar was added to some dishes immediately (zero time). The remaining Petri dishes received 5 ml. 10% normal rabbit serum in Hanks solution and were kept at 37°.

At different intervals, dishes were washed twice with Hanks solution and melted trypticase soy agar was added. The zero time was normally some 90 min. after initial phagocytosis of organisms by leucocytes. By this method, if intracellular killing of organisms should occur during the period of observation, plate counts at intervals should decrease, provided that the population of leucocytes per Petri dish remained unchanged.

Continued phagocytosis of extracellular organisms would also give the impression that killing was occurring, but since such small numbers were used, the possibility of this phenomenon is remote.

RESULTS

The first logical approach was to check whether the intracellular behaviours of the Smith strain and 18-Z strains were different. The method described by Cohn & Morse was used to follow this.

Strain 18-Z and rabbit monocytes. Figure 1 illustrates the results of such an experiment in which 10% normal and specific immune sera were used. It appeared that the decline of the total bacterial count of the 18-Z strain was comparable to that of the Smith strain as reported by Mackaness (1960).

Strain 18-Z and rabbit polymorphs. A series of experiments was performed to follow the intracellular survival of 18-Z in rabbit polymorphs using normal rabbit serum. At the same time it was decided to see if repeated injections of glycogen intraperitoneally into rabbits might perhaps change the activity of the leucocytes from the corresponding exudates. Therefore, 150 ml. of 0·1% glycogen in pyrogen-free saline was injected intraperitoneally, daily into a group of rabbits. Four hr. after the glycogen injection 100–150 ml. of Hanks solution with heparin (1/20,000) was injected into the peritoneal cavity and the exudate collected through a trocar. Figure 2 illustrates data obtained with polymorphs from a representative rabbit. It can be seen that the intracellular survival of 18-Z in rabbit polymorphs is comparable to that observed in rabbit monocytes, but is quantitatively different from that reported for the Smith strain by Cohn & Morse (1959). Furthermore, although the number of polymorphs obtainable in the exudate increased with repeated glycogen stimulation, the intracellular behaviour of the organisms appeared to remain essentially the same in cells from the same rabbit.

Smith strain in rabbit polymorphs. The survival of the Smith strain within polymorphs from different rabbits was studied with specific immune serum. Figure 3 illustrates the result of such an experiment. The survival of the Smith strain within

polymorphs was similar to that observed with the 18-Z strain within polymorphs and monocytes. It was not possible to demonstrate the rapid and extensive destruction of the organisms as reported by Cohn & Morse (1959).

An attempt was also made to follow the fate of the 18-Z and Smith strains within rabbit monocytes shortly after phagocytosis by using the plastic chamber method previously described. Here, only 15 min. were allowed for the settling of the monocytes on to the coverslip; the zero time samples were actually taken some 20 min. after the initial contact of monocytes and organisms. These experiments were performed with normal rabbit serum in the absence of antibiotics. Figure 4A shows the results of such an experiment in which there was no apparent destruction of the intracellular 18-Z strain and Smith strain up to 3 hr. It also confirmed that the 18-Z strain was phagocytosed in normal rabbit serum while the Smith strain could resist phagocytosis under the same conditions.

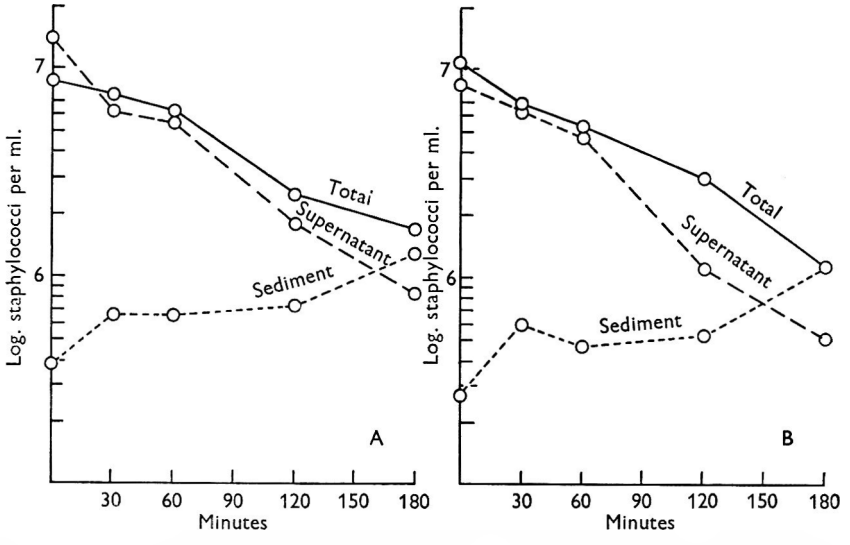

Fig. 1. The survival of *Staphylococcus aureus* 18-Z within rabbit monocytes. A. In the presence of normal rabbit serum. B. In the presence of specific immune rabbit serum.

Packing the leucocytes and organisms by centrifugation (described under the plate method) was found to be effective in aiding the initial phagocytosis of the Smith strain. This avoided the use of antiserum whose presence might interfere with the results, hence the packing method in the presence of normal rabbit serum was routinely used. Figure 4B illustrates the result of such an experiment which was performed in the presence of 20 μg. streptomycin/ml. medium, and using an 18 hr. old culture of the Smith strain. Here there is some indication of slight intracellular destruction of the organism, but this does not appear to be dependent upon specific antibodies. Perhaps this slight destruction is in part due to the presence of streptomycin, since the Smith strain is more sensitive to the antibiotic than is the 18-Z strain.

In order to follow the intracellular survival of staphylococci for a somewhat longer period, without resorting to antibiotics as a means of preventing extracellular multiplication, experiments were also performed using the adenine-requiring and strepto-

mycin-dependent mutants of strain 18-Z. Monocytes were infected with these mutants and placed in plastic chambers in the absence of any antibiotics. Figure 5 presents data from such studies over a 10 hr. period. There is no evidence of any significant destruction of these mutants within monocytes during this interval.

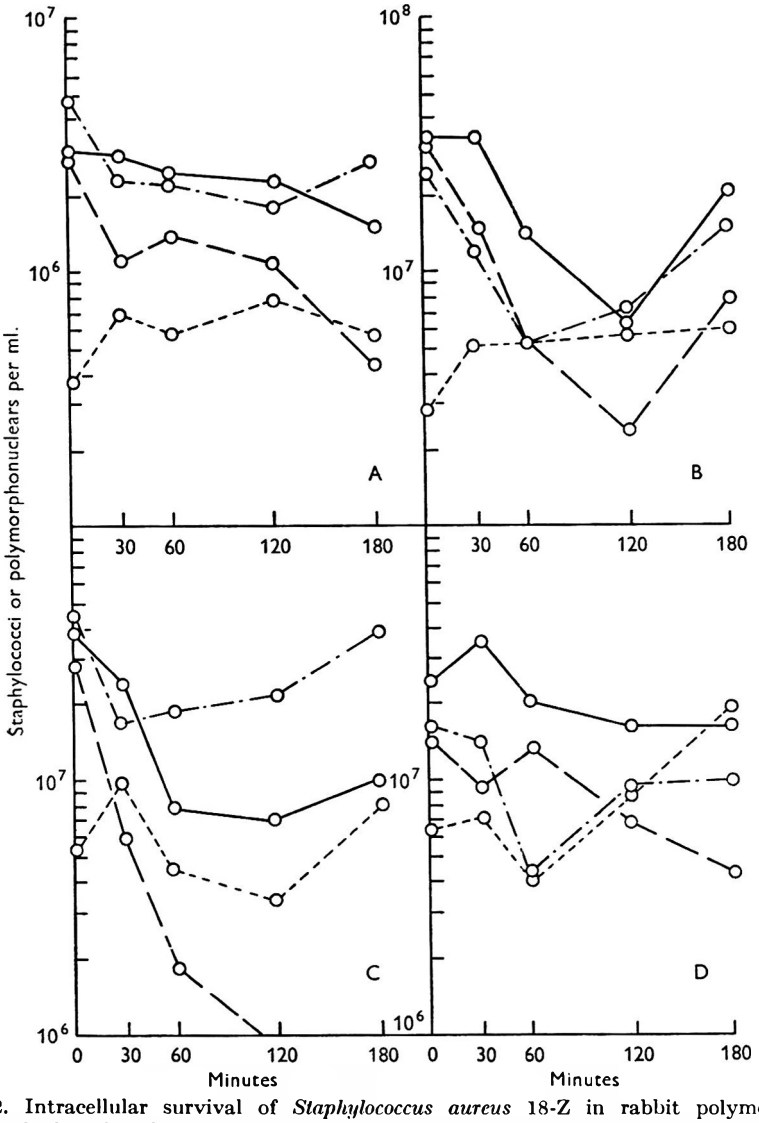

Fig. 2. Intracellular survival of *Staphylococcus aureus* 18-Z in rabbit polymorphs obtained 4 hr. after the first (A), third (B), fifth (C), and eighth (D) daily intraperitoneal glycogen injection. — Total; --- sediment; — — supernatant; —·—·— polymorphs.

The plate method offered a different approach to this problem. Rabbit polymorphs and monocytes were infected with the 18-Z and Smith strains as described under Methods. The results are illustrated in Fig. 6 in which there appeared to be little, if any, intracellular destruction of the 18-Z or Smith strain within rabbit polymorphs or monocytes during the period of observation.

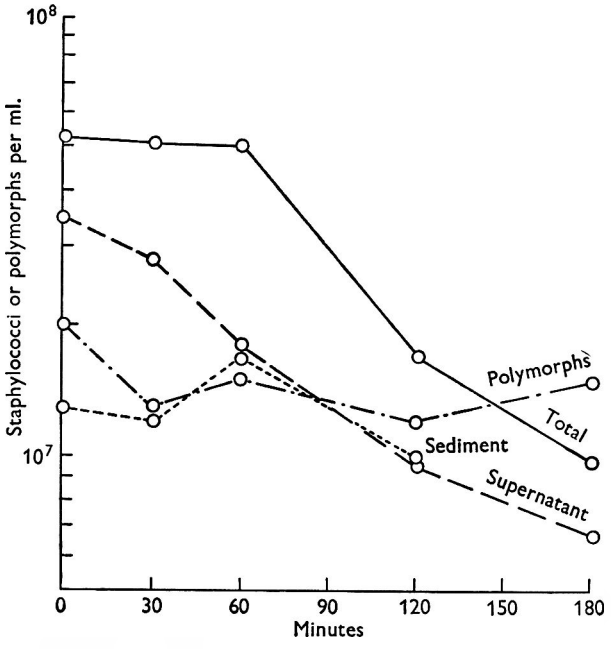

Fig. 3. The intracellular survival of *Staphylococcus aureus* Smith within rabbit polymorphs in the presence of specific antiserum.

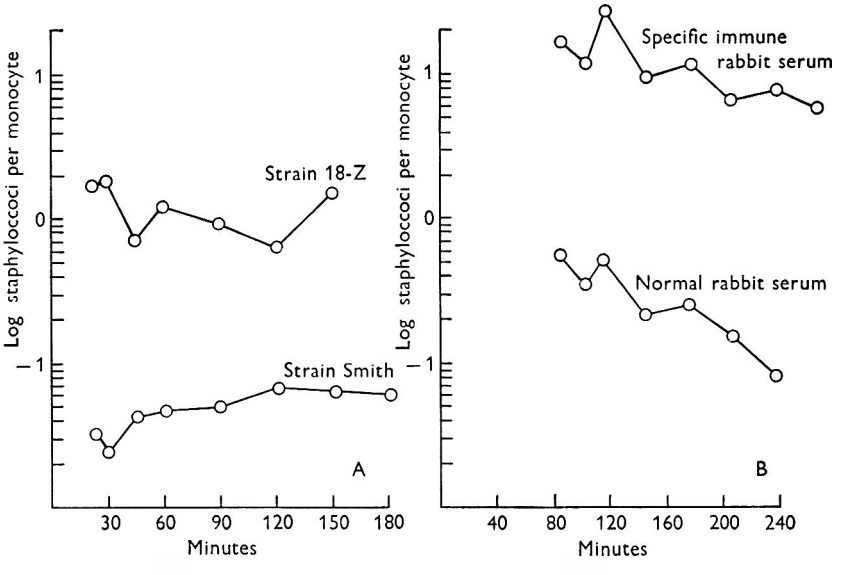

Fig. 4. A. Intracellular survival of *Staphylococcus aureus* Smith and 18-Z within rabbit monocytes (no antibiotic in medium). B. Intracellular survival of *S. aureus* Smith within rabbit monocytes in the presence of normal and immune rabbit serum (20 μg. streptomycin/ml. medium).

The suspended tissue culture method was also employed to study the immediate fate of intracellular staphylococci. In some experiments, the period of observation was extended up to 10 hr. With this method, the intracellular, extracellular, and total population of organisms as well as the viable leucocytes could be followed. With the Cohn & Morse method the rapid decline of the total population and the extracellular organisms was interpreted as the combined effect of continuous phagocytosis and intracellular destruction. In order to eliminate continuous phagocytosis of extracellular organisms which might complicate determining the fate of intracellular organisms, it would be desirable to remove as many extracellular staphylococci as possible subsequent to initial infection of the cells. The continuous flow centrifugation procedure as described under Methods was used and the period from initial phagocytosis to removal of zero time samples was reduced to 18–20 min.

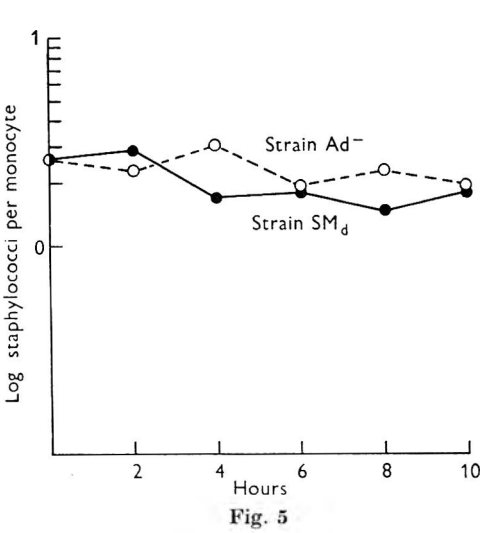

Fig. 5

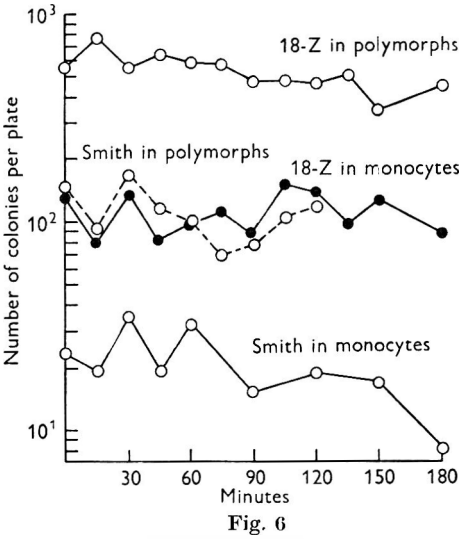

Fig. 6

Fig. 5. Intracellular survival of adenine-requiring (Ad^-) and streptomycin-dependent (SM_d) mutants of *Staphylococcus aureus* 18-Z within rabbit monocytes maintained in plastic chambers.

Fig. 6. Survival of *Staphylococcus aureus* 18-Z and Smith strains within rabbit polymorphonuclear leucocytes and monocytes by the plate method.

The number of leucocytes in successive samples sometimes decreased for a period, and then again increased after 3–5 hr. This apparently did not reflect a change in total cell population within the flasks since cells were observed to settle along the walls of the vessel. However, such decreases did not occur in all experiments and did not depend on infection since the phenomenon was also observed with non-infected leucocyte cultures (but was not observed with heat killed leucocytes). Apparently the cells adhered to the vessel walls for a time, but later released themselves into the medium. The same phenomenon often occurred in siliconized test tubes (using the Cohn & Morse procedure); thus the shape of the vessel did not appear responsible for the effect. The mechanism for this reaction is, therefore, not clear.

A series of experiments were performed with rabbit polymorphs or monocytes infected with 18-Z, 18-Z SM_d and Smith strains. A different rabbit was used as a leucocyte donor in each experiment.

Figure 7A illustrates the results obtained when rabbit monocytes were infected with a washed 6 hr. old culture of 18-Z. Fifty μg. streptomycin/ml. were added to the medium 1 hr. after the zero time sampling, in order to suppress extracellular multiplication of staphylococci.

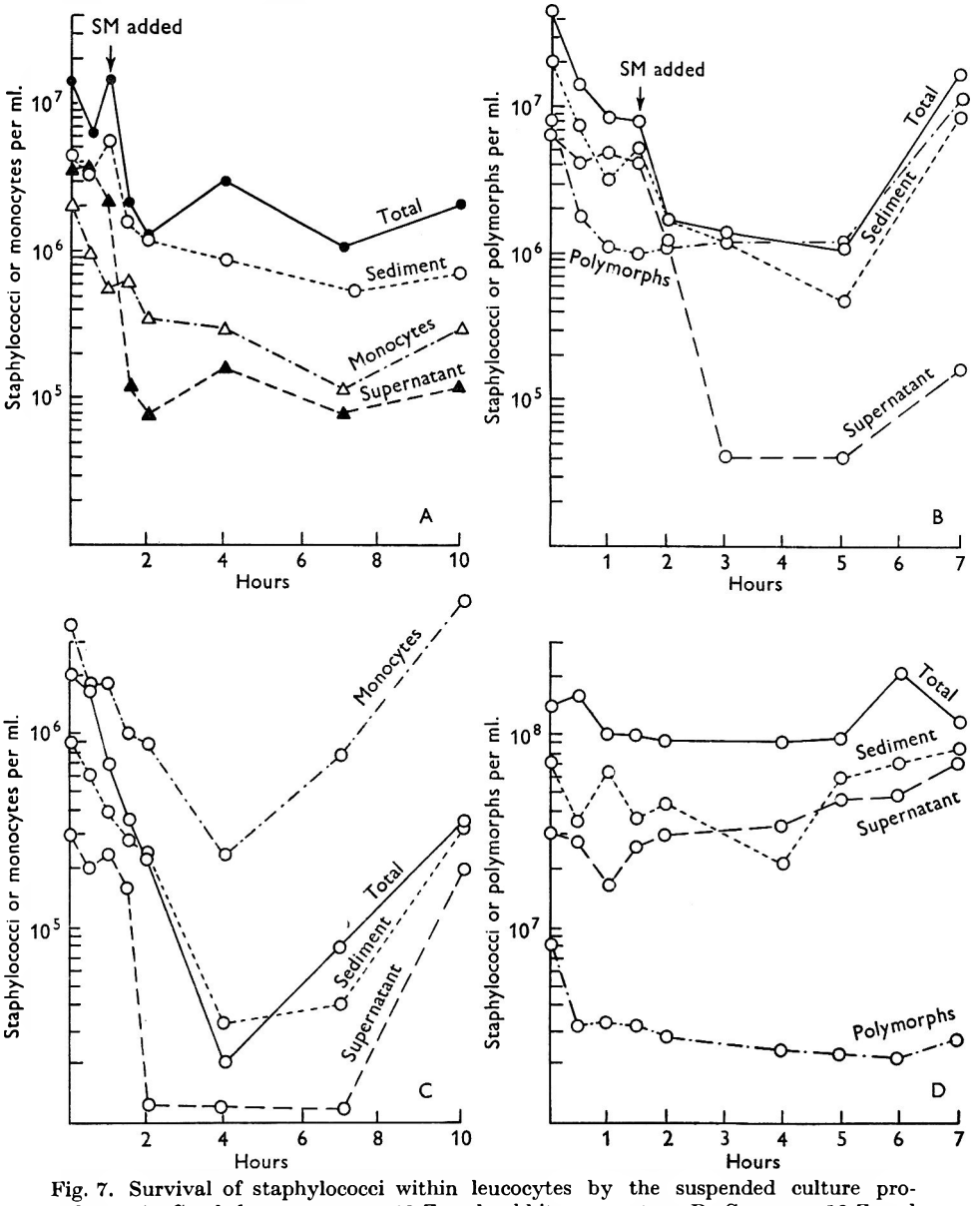

Fig. 7. Survival of staphylococci within leucocytes by the suspended culture procedure. A. *Staphylococcus aureus* 18-Z and rabbit monocytes. B. *S. aureus* 18-Z and rabbit polymorphs. C. *S. aureus* 18-Z SM_d and rabbit monocytes. D. *S. aureus* 18-Z SM_d and rabbit polymorphs. SM = streptomycin.

Figure 7B shows the results of an experiment in which a polymorph suspension was infected with a 16 hr. culture of 18-Z, 50 μg. streptomycin/ml. was added to the suspended culture medium 1·5 hr. later.

Figure 7C illustrates the result of an experiment in which rabbit monocytes were infected with a 6 hr. culture of the streptomycin-dependent mutant of 18-Z (18-Z, SM_d). Figure 7D shows the findings when rabbit polymorphs were infected with a 16 hr. culture of the same organism.

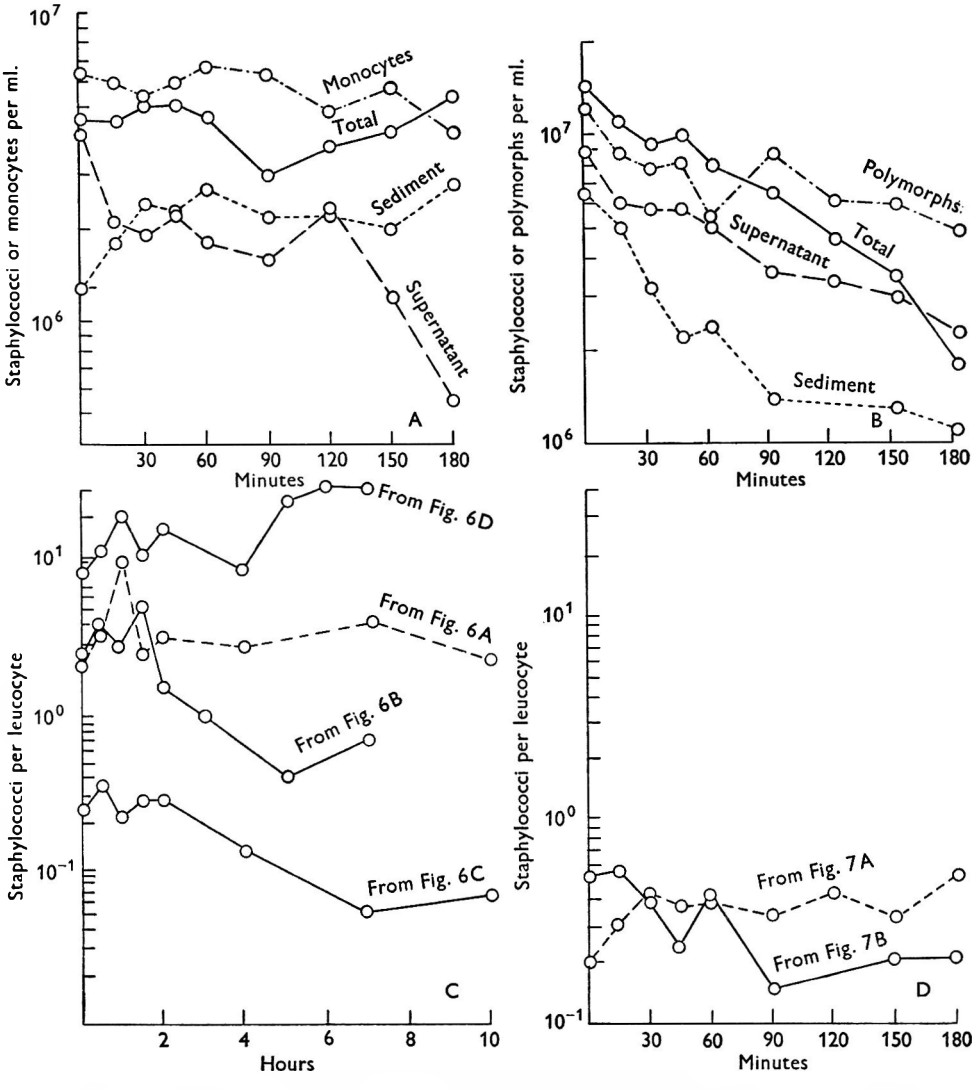

Fig. 8. A. Survival of *Staphylococcus aureus* Smith within rabbit monocytes. B. Survival of *S. aureus* Smith within rabbit polymorphs. C. Average number of organisms (*S. aureus* 18-Z and 18-Z, SM_d) per cell from data plotted in Fig. 6A–D. D. Average number of organisms (*S. aureus* Smith) per cell from data plotted in Fig. 7A–B.

Figure 8A and B illustrate the results of two experiments in which rabbit monocytes or polymorphs were infected with a 20 hr. culture of the Smith strain of *Staphylococcus aureus*, and the infected cell cultures maintained for 3 hr. in the absence of antibiotics.

In the experiments illustrated by Fig. 7B and C, it can be seen that the leucocyte counts decreased temporarily and later increased to the original values during the course of study. However, it may be noted that the number of organisms in the sediment and the total staphylococcal population paralleled the leucocyte population fairly well. Thus the average number of staphylococci per cell (sediment count/leucocyte count) in the cultures usually remained relatively constant for several hr. This is demonstrated in Fig. 8C and D where the average number of staphylococci/cell is plotted against time for all the previous mentioned experiments using the suspended culture technique.

DISCUSSION

Four methods were used to follow the fate of intracellular staphylococci, each method having its advantages and limitations. In the tissue-culture chamber method, the infected leucocytes adhered to cover slips in the same chamber, and the populations of intracellular and extracellular organisms and that of the leucocytes could be followed. However, this method does not permit an independent evaluation of the total population of organisms nor does it allow accurate determination of the intracellular population in the presence of streptomycin during the first hour after phagocytosis.

The Cohn & Morse method, in addition, permitted the enumeration of the total population of organisms, but continuous phagocytosis in this method might complicate determining the intracellular fate of the organisms.

The suspended culture method is a modification of the Cohn & Morse method. This procedure had all the advantages of the chamber method and Cohn & Morse method and in addition made possible sampling of intracellular organisms within 20 min. after phagocytosis in the absence of an excessive extracellular population. Also the packing of leucocytes and organisms permitted rapid phagocytosis without using antibodies. It also allowed all samples to be derived from one vessel. Enumeration of viable leucocytes in each sample gave an opportunity to evaluate the staphylococcal counts in relation to the leucocyte counts.

The plate method has the advantage that it so separates the leucocytes from the extracellular organisms that continual phagocytosis is virtually impossible even without antibiotics.

The results obtained using these methods indicated that *Staphylococcus aureus* (18-Z and Smith strains) survive in significant numbers within normal rabbit monocytes and polymorphs for several hours. These methods failed to demonstrate any extensive immediate destruction, as reported by Cohn & Morse (1959).

Rogers & Melly (1960), using the same procedure and the same organisms, but using normal human blood leucocytes, did not report the same degree of killing as presented in Cohn & Morse's work. Melly *et al.* (1960) further studied the intracellular staphylococci by direct observations with phase contrast microscopy and demonstrated some intracellular destruction of staphylococci, but not as much as found by Cohn & Morse. These latter studies were performed using the leucocytes from a single human donor and thus might not reflect a general phenomenon. This view might be supported by the observed variation in the behaviour of staphylococci within leucocytes derived from different rabbits (Shayegani & Kapral, 1962).

It is still difficult at the present time to evaluate the role of leucocytes in the pathogenesis of staphylococcal disease. It appears that the usual *Staphylococcus aureus* strains (represented by the 18-Z strain) are readily phagocytosed in the presence of normal serum whereas encapsulated strains (such as the Smith strain) may present such an unusual surface to the leucocytes that phagocytosis is reduced in the absence of specific antibodies. What has been termed as normal serum in this study may, however, still contain opsonizing antibodies against *S. aureus*. The possibility exists that there is a widespread occurrence in the serum of many rabbits of antibodies against certain surface antigens commonly associated with *S. aureus* while antibodies against uncommon capsular antigens may be less frequently encountered. The recent report by Cohen, Cowart & Cherry (1961) demonstrates that antibodies against *S. aureus* are indeed frequently found in the serum of non-immunized rabbits. Furthermore, it should be mentioned that more recent studies by Dr Isabel Li in this laboratory have demonstrated a more pronounced destruction of *Staphylococcus aureus* phagocytosed in the presence of certain specific immune sera than reported in this publication. Studies concerned with the possibility that variations in antisera may account for some of the published quantitative discrepancies are now under way. Nevertheless, after phagocytosis the question still arises whether leucocytes *in vivo* can effectively destroy *Staphylococcus aureus* under conditions existing locally at the site of infection. Further work is necessary to answer this question.

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The Eventual Intracellular Destruction of Staphylococci by Mononuclear Cells

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SUMMARY

It was found that *Staphylococcus aureus* usually survived within monocytes of normal rabbits for several hours without multiplication, but were eventually destroyed. However, there was variation in the intracellular behaviour in leucocytes of different rabbits in that cells from some donors began the slow destruction of the staphylococci shortly after phagocytosis.

In many of these experiments streptomycin was incorporated in the tissue-culture medium to suppress the extracellular multiplication of staphylococci. Interference by streptomycin with the intracellular behaviour of staphylococci was considered as minimal on the basis that the intracellular survival was not influenced by different concentrations of the drug in the tissue-culture medium nor did incubation of the cells in the antibiotic before infection alter the subsequent survival of the organisms.

INTRODUCTION

In a previous study (Kapral & Shayegani, 1959) it was reported that *Staphylococcus aureus* could survive for several hours within rabbit mononuclear leucocytes. No multiplication or destruction of staphylococci was observed during this interval with the methods used. Subsequently attempts were made to determine the eventual fate of the surviving intracellular staphylococci.

METHODS

Staphylococcal strain. *Staphylococcus aureus* strain 18-Z; the characters of this strain were described by Kapral & Shayegani (1959). A mutant strain 18-Z, SM_r resistant to 1000 μg. streptomycin/ml. medium was isolated from the parent 18-Z strain. The mutant appeared identical with the parent strain in all other *in vitro* characteristics.

Monocytes. Rabbit mononuclear exudate cells were prepared as described by Kapral & Shayegani (1959).

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Tissue culture procedures

Plastic chamber method. This tissue-culture procedure was the same as previously described (Kapral & Shayegani, 1959) with the exception that 0·2 ml. of 10% sodium bicarbonate solution was incorporated into the tissue-culture medium and the chambers placed under 5% CO_2 to stabilize the pH at 7·4 during the long incubation period. Fifty μg. streptomycin/ml. medium was used in these experiments.

Suspended cultures. Suspensions of mononuclear leucocytes and *Staphylococcus aureus* 18-Z were prepared as previously described (Kapral & Shayegani, 1959). About 2×10^6 monocytes and 5×10^9 staphylococci were suspended in 1 ml. of 10% normal rabbit serum in Hanks solution. The mixture was centrifuged at 100*g* for 3 min., then placed in a water bath at 37° for 5 min. The sedimented infected cells were washed three times with 10 ml. quantities of cold Hanks solution containing 10% normal rabbit serum. Samples of the washed infected cells were then placed in siliconized 50 ml. flasks containing 10 ml. of 10% normal rabbit serum in Hanks solution at pH 7·2. The suspensions were kept agitated by Teflon stirring bars driven by a magnetic stirrer. After 1 hr. incubation streptomycin was added to medium to give a final concentration of 50 μg./ml. Samples were removed and treated according to Cohn & Morse (1959) with the exception that the cells were lysed by 5% saponin solution and that counts of viable (trypan blue negative) leucocytes were also done on each sample.

RESULTS

A series of experiments was performed with normal rabbit monocytes infected with *Staphylococcus aureus* 18-Z in plastic tissue-culture chambers.

Figure 1 illustrates some representative results. There was considerable variation in the intracellular survival of *S. aureus* 18-Z within monocytes of different rabbits. In one case (A) there was a persistent survival for 30 hr. In another instance (B) there was a period of survival followed by destruction. In still other rabbit monocytes (C, D) staphylococci were destroyed without any evidence of a period of survival.

In many experiments the serum of the rabbit donating the cells as well as the rabbit serum used in the tissue-culture medium was tested for the presence of antibody against soluble products of the test organism by the method of Elek & Levy (1950). In these cases no detectable precipitating antibodies were found; therefore, the rabbits were assumed to be normal.

Different multiplicity of infection. Since it was possible that the late destruction of strain 18-Z might somehow be dependent on the number of organisms per cell, experiments were performed with different multiplicities of infection using cells from the same rabbit. It was noted that the eventual destruction of intracellular staphylococci did not depend on the multiplicity of infection. This is illustrated in Fig. 2 where multiplicities of 25 and 100 staphylococci per monocyte were used to yield different initial infectivities, yet the fate of the organisms was the same in both instances.

Destruction within monocytes maintained in suspension. Studies using suspended

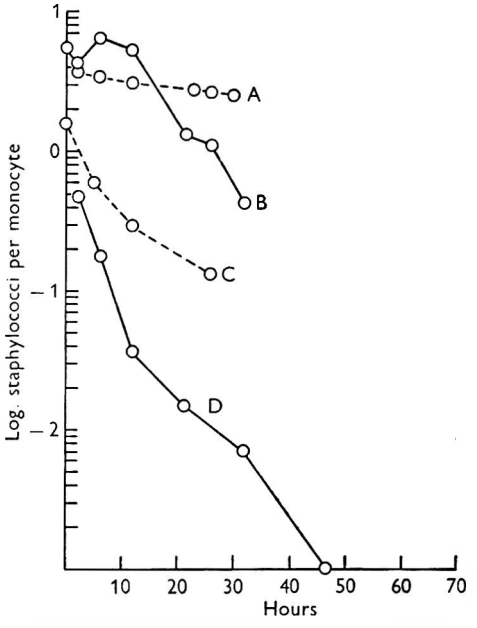

Fig. 1. Long-term survival of *Staphylococcus aureus* strain 18-Z within monocytes derived from different rabbits (A–D).

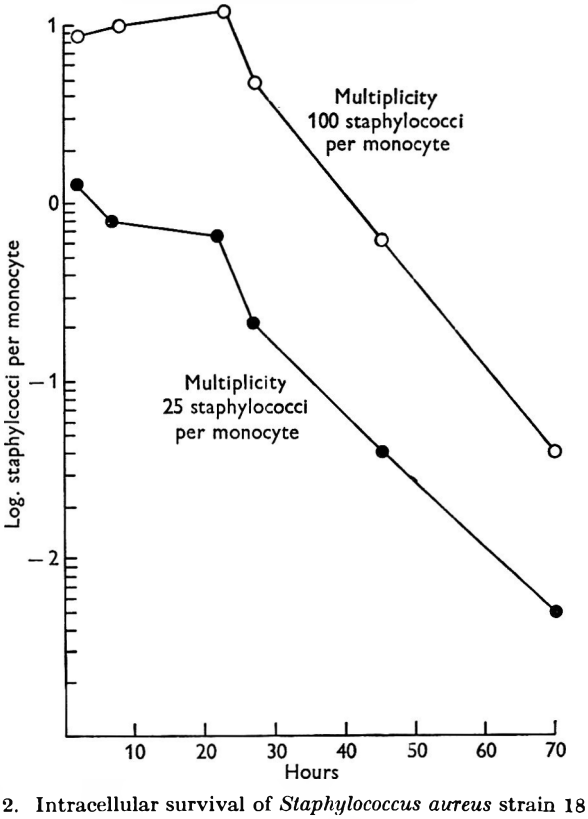

Fig. 2. Intracellular survival of *Staphylococcus aureus* strain 18-Z with different multiplicities of infection.

cultures gave similar results as obtained in the plastic chambers. Fig. 3 illustrates the results obtained in an experiment where infected monocytes were maintained for 50 hr. After a short period of survival the staphylococci were slowly destroyed.

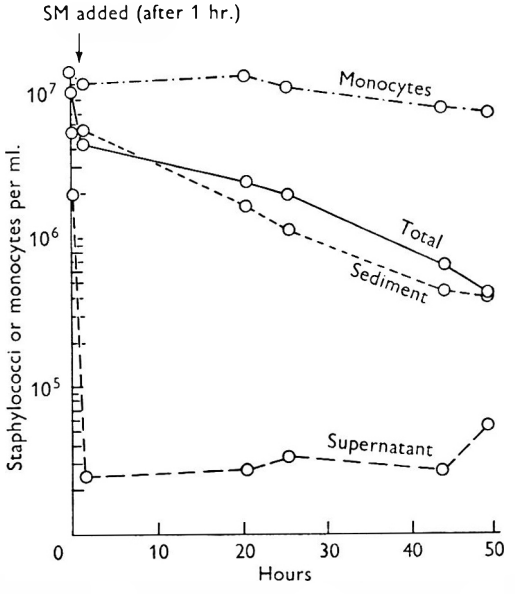

Fig. 3. Survival of *Staphylococcus aureus* strain 18-Z within rabbit monocytes maintained in suspension.

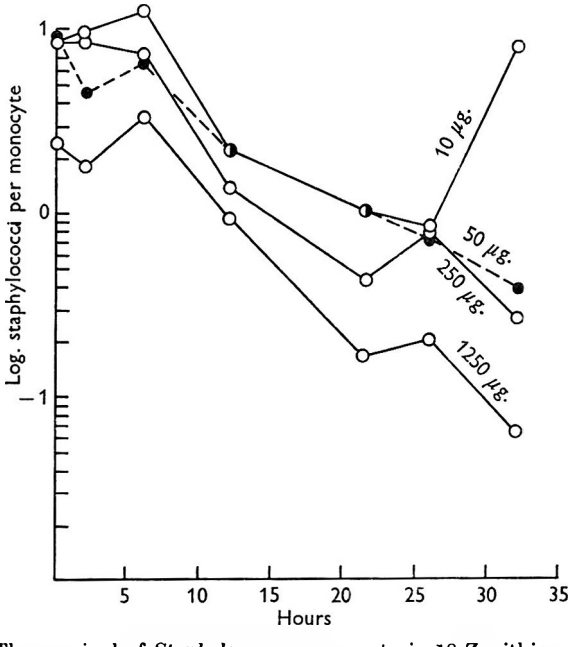

Fig. 4. The survival of *Staphylococcus aureus* strain 18-Z within monocytes in the presence of various concentrations of streptomycin.

Since streptomycin was routinely incorporated into the tissue-culture medium, there was a possibility that this antibiotic might penetrate into the monocytes, which could in turn account for the intracellular destruction of staphylococci.

The following experiments are reported since the data tend to indicate that streptomycin does not seriously interfere with the intracellular behaviour of staphylococci.

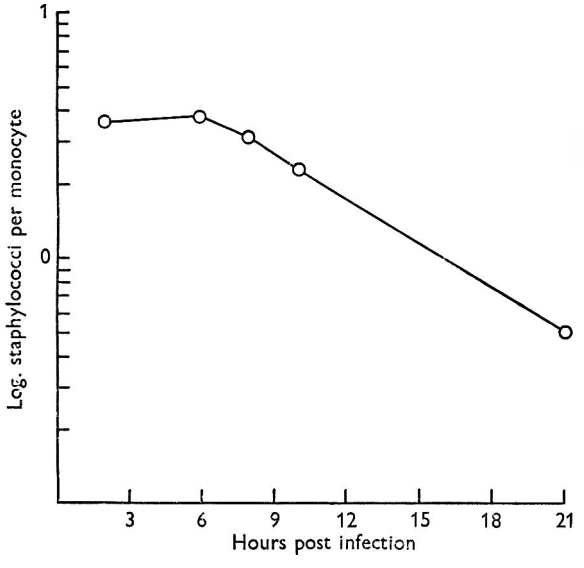

Fig. 5

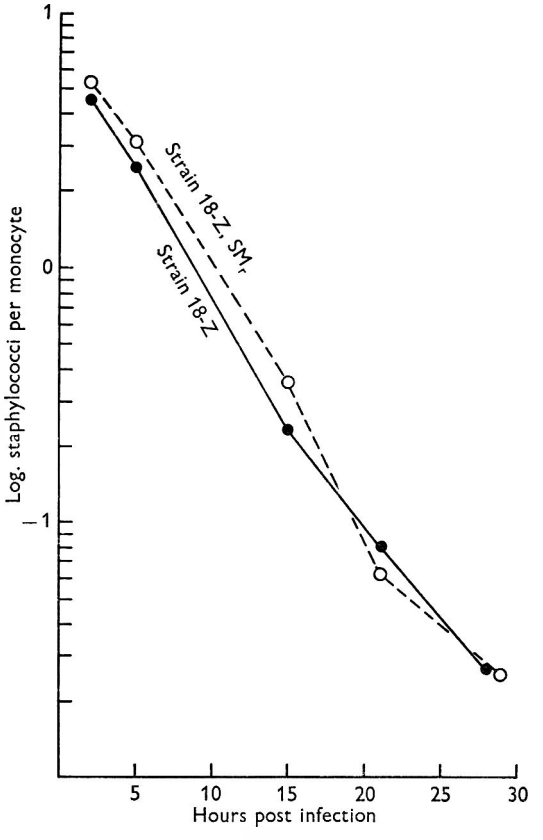

Fig. 6

Fig. 5. Survival of *Staphylococcus aureus* strain 18-Z within monocytes maintained in the presence of streptomycin before infection.

Fig. 6. Survival of *Staphylococcus aureus* within monocytes maintained in the presence of streptomycin before infection with strain 18-Z, SM_r.

Different concentrations of streptomycin in tissue-culture medium. A series of experiments was performed with monocytes from one rabbit to compare the effect of different concentrations of streptomycin in the tissue-culture medium on the rate of destruction of intracellular staphylococci. It seemed reasonable to assume that if the streptomycin would slowly penetrate into the monocytes, a higher concentration in the tissue-culture medium would increase the rate of penetration and perhaps increase the rate of killing of intracellular staphylococci. Fig. 4 illustrates the results obtained under the conditions in which 10, 50, 250 and 1250 μg. streptomycin/ml. medium were used. Under these conditions the intracellular destruction

in the presence of 10–250 μg./ml. was essentially the same. It should be noted, however, that after 26 hr., streptomycin in a concentration of 10 μg./ml. medium no longer suppressed the extracellular multiplication of *S. aureus* 18-Z. It was because of such failures of low concentrations of streptomycin reliably to control the extracellular population of the strain that higher concentrations were routinely used. When 1250 μg. streptomycin/ml. medium was used, there appeared to be a slightly greater decline in the intracellular staphylococci, but it is difficult to say whether this is significant.

Delayed infection of monocytes. It was also thought that if the intracellular staphylococci were destroyed by the slow penetration of streptomycin rather than by the innate capacity of the monocytes, it might result in increased killing if the monocytes were allowed to equilibrate with streptomycin before infection.

It was found that monocytes adhering to coverslips in tissue-culture chambers for some 24–30 hr. (in medium containing 50 μg. streptomycin/ml. medium) could readily phagocytize organisms which were introduced into the chamber.

Fig. 5 shows that the behaviour of staphylococci within monocytes kept in medium containing streptomycin for 24 hr. before infection was similar to that found if phagocytosis had occurred immediately.

Infection with streptomycin-resistant mutant. Monocytes from one rabbit were placed in tissue culture in the presence of 50 μg. streptomycin/ml. for 30 hr. At this time the cells were infected with either strain 18-Z or the streptomycin-resistant strain 18-Z SM_r. Streptomycin (50 μg./ml.) was present in the medium in both cases, but in cultures infected with the streptomycin-resistant mutant, 40 μg. Kanamycin/ml. was also added to control extracellular multiplication. It will be seen (Fig. 6) that these strains behaved similarly within the cells; therefore, it is not likely that the killing was due to the presence of streptomycin within the leucocytes. However, since Kanamycin was used to control the extracellular streptomycin-resistant mutants a possibility remains that the leucocytes became 'permeable' to both drugs after a time and the destruction actually resulted from the antibiotics. It may also be noticed that the organisms were killed early and continually in these particular cells.

DISCUSSION

Tissue-culture methods offer an experimental approach to the study of intracellular bacterial pathogens. However, the conditions are such that extracellular multiplication of the organisms can be misinterpreted as intracellular multiplication if continuous phagocytosis by the host cells proceeds. Antibiotics (e.g. streptomycin) were incorporated into the tissue-culture medium in order to reduce this possibility by inhibiting extracellular multiplication. However, the possibility remains that the antibiotic so used may penetrate the host cells and thereby become bactericidal or bacteriostatic for intracellular organisms. There is no obvious direct experimental method to disprove such a possibility. It appears very likely that streptomycin enters leucocytes by means of pinocytosis, but this does not necessarily imply that the antibiotic can actually come into contact with the intracellular organism. The membrane about the vacuoles might still prevent penetration of the

antibiotic into cytoplasm or the vacuole containing the organism. However, indirect experiments were performed to determine any gross effects of streptomycin on the behaviour of the intracellular staphylococci. The removal of most of the extracellular organisms by repeated washing gave results similar to those when streptomycin was used, but this procedure was impractical for long periods. Furthermore, the intracellular behaviour was not critically dependent on the streptomycin concentration in the medium, nor did pre-incubation of cells in the antibiotic alter the fate of the phagocytized staphylococci. The fact that tubercle bacilli and brucella can multiply within monocytes in the presence of streptomycin (Suter, 1953; Hsu & Kapral, 1960; Berthrong & Hamilton, 1959; Pomales-Lebron & Stinebring, 1957; Murate, Stinebring, Schaffner & Lechevalier, 1959) lends support to the idea that this antibiotic does not drastically affect intracellular organisms.

The results reported here also indicate that the intracellular behaviour of staphylococci may differ in leucocytes from different rabbits. Since no detectable precipitating antibody was demonstrated in the sera of these animals it was presumed that these rabbits had had no recent major contact with the organism. This variation suggests the possibility that there may be an inherent difference in the leucocytes from different donors, but the variation might also result from subtle differences in the manipulation of the cell preparations.

The prolonged intracellular survival of *Staphylococcus aureus* might explain part of the pathogenesis of staphylococcal infections. Kapral & Li (1960) and Li & Kapral (1962) showed that *S. aureus* 18-Z (and derived coagulase negative mutants) when injected intravenously into rabbits survived (without apparent multiplication) in the lungs, liver, and spleen for as long as 2–3 weeks. It is possible that the organisms persist within reticulo-endothelial cells of these organs, but proof of this is lacking.

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The Mechanism of Lysis of *Prymnesium parvum* by Weak Electrolytes

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SUMMARY

Organisms of the phytoflagellate *Prymnesium parvum* Carter undergo swelling and lysis in the presence of ammonia or acetic acid. The lytic activity was pH-dependent and increased as the concentration of undissociated weak electrolyte in the suspension medium was increased. The kinetics of swelling were followed with the aid of an electronic particle counter and by microscopic examination. In the presence of ammonia or acetic acid, the rate of swelling as well as the final volume of the Prymnesium organisms were a function of external osmotic pressure and of temperature. It is suggested that the swelling and lysis are osmotic in nature and depend on the intracellular accumulation of weak electrolytes by a 'pump' driven by differences between environmental and intracellular pH values. The similarities between this phenomenon and concentration of weak electrolytes in mammalian erythrocytes and other cells is discussed. Differences in the morphology and lysis of Prymnesium by ammonia and acetic acid were found which suggest the existence of intracellular compartments maintained at different pH values.

INTRODUCTION

Prymnesium parvum Carter, a phytoflagellate of the Chrysomonadinae has become the object of much study because of its ability to form toxic material which kills fish (Otterstroem & Steeman-Nielsen, 1939; Reich & Aschner, 1947; Shilo & Aschner, 1953; McLaughlin, 1956; Shilo & Rosenberger, 1960; Yariv & Hestrin, 1961). Reich & Aschner (1947) observed the extreme sensitivity of Prymnesium to ammonium sulphate and focused attention on the use of this salt to control the phytoflagellate in fishponds. In the present paper the effect of environmental conditions on the lytic activity of weak electrolytes on Prymnesium has been studied and the sequence of morphological changes which take place during lysis has been examined. Recent advances in cell-sizing by use of an electronic particle counter made it possible to follow the kinetics of swelling and lysis of Prymnesium populations by penetrating weak electrolytes. On the basis of these results a mechanism which concentrates weak electrolytes in the interior of Prymnesium cells and which may lead to osmotic lysis has been suggested.

METHODS

Prymnesium parvum cultures. Two strains of *P. parvum* grown in axenic culture were used. (1) A strain isolated from brackish water fishponds in Israel by K. Reich, (Dept. of Zoology, Hebrew University, Jerusalem; Reich & Kahn, 1954). (2) A strain isolated from supralittoral pools in Scotland by M. R. Droop (Marine Station, Millport, Great Britain; Droop, 1954). These organisms will be referred to as Prymnesium. *Dunaliella salina* Teodor. and *Syracosphaera carterae* from the stock collection of the Department of Botany, Hebrew University of Jerusalem were kindly given us by Dr I. Friedman.

Media and conditions of growth. Throughout this work a modification of a medium developed by Dr M. R. Droop (personal communication) for growth of marine phytoflagellates was used. It was composed of two equal parts, (A) sea water 25 % (v/v) and (B) (%, w/v): liver extract (Difco), 0·02; glucose, 0·02; Bacto-tryptone (Difco), 0·02; NaCl, 0·75; $MgCl_2.6H_2O$, 0·5; KCl, 0·08; $CaSO_4$ (anhydrous) 0·1; in distilled water; pH 8. In some experiments the osmotic pressure of this medium was changed by altering the concentration of sea water (1·5–50 %, v/v) and of sodium chloride (0·1–3 %, w/v). Sterilization of media was performed by autoclaving at 120° for 20 min. Cultures were grown in Erlenmeyer flasks of 250 ml. capacity containing 100 ml. liquid medium in continuous 'fluorescent white' daylight, 220–260 foot candles at 26°–28°. Under these conditions populations of 3 to 5×10^6 organisms/ml. were reached within 4–6 days.

Measurements of organism volume. For the measurement of the volumes of Prymnesium organisms, the Coulter Particle Counter, Model A (Coulter Electronics, Chicago, Illinois, U.S.A.) with an orifice of 100 μ was used. This instrument provides a means to determine size distribution of cell populations and has already proved extremely useful in the counting and sizing of erythrocytes (Mattern, Brackett & Olson, 1957), in the estimation of the volumes of cells in tissue cultures (Kuchler & Merchant, 1958; Brecher, Schneiderman & Williams, 1956) and of bacteria (Kubitschek, 1958; Lark & Lark, 1960).

The instrument applies principles of electrical gating. The cells are suspended in an electrically conductive medium and forced to flow through a small aperture having an immersed electrode on each side. As each particle passes through the aperture it replaces its own volume of electrolyte, momentarily changing the resistance value between the electrodes. This change produces a voltage pulse of short duration having a magnitude proportional to particle volume and the resultant series of pulses are electronically amplified, scaled and counted.

Samples of Prymnesium cultures for counting and sizing were diluted in the growth medium and carefully filtered to give low background values. Population densities of 40,000–60,000 organisms/ml. were used, since at this concentration the coincidence effect of more than one organism passing through the orifice at the same time was comparatively small (5–7·5 %).

Lysis and swelling in Prymnesium populations under various experimental conditions were estimated by measurement of the size distribution of organisms in populations at different times. Organism size distribution in a single population followed a typical normal distribution curve (Fig. 1). The threshold value corresponding to 50 % of the population (corresponding to the maximum of the Gaussian

distribution curve) was a convenient measure of the size characteristics of the population and has been used in the expression of our results. This measure has been given in 'threshold units' of the counter which, under standard conditions, are directly proportional to volume. This relationship was established experimentally with erythrocytes of various mammalian species (Fig. 2). The erythrocytes were suspended in the Prymnesium growth medium isotonic for these cells and the

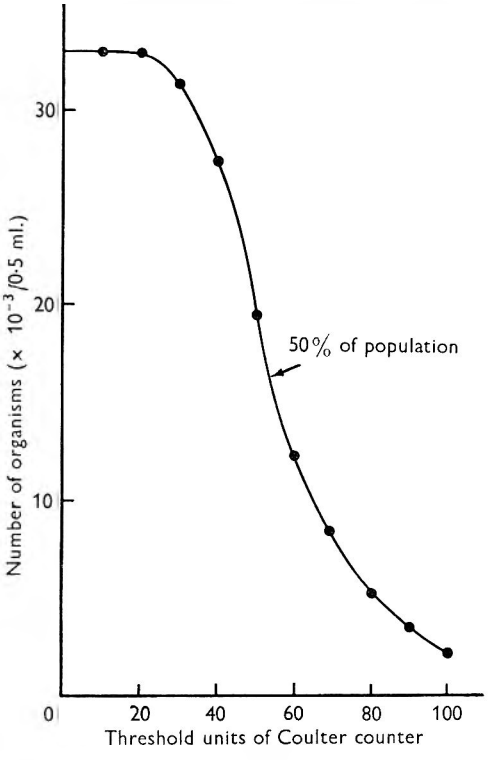


Fig. 1

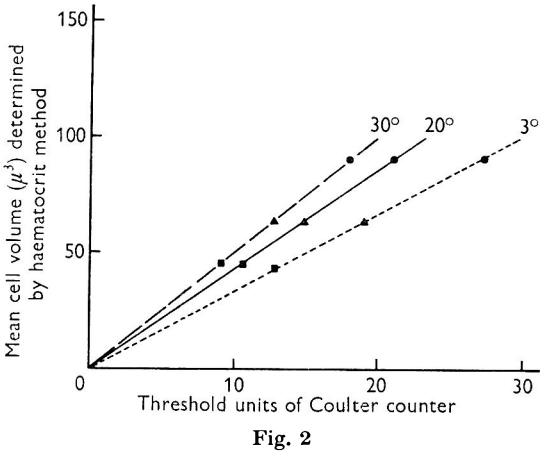


Fig. 2

Fig. 1. Size distribution of Prymnesium organisms as measured in the Coulter electronic particle counter. Organisms of a 2-day culture were suspended in Prymnesium growth medium, counted and sized at 30°. Readings were taken at the following settings of the Coulter counter: gain switch, 3; gain trim, 7; aperture current setting, 5.

Fig. 2. The relation between mean cell volume of various mammalian erythrocytes and 'threshold readings' in the Coulter counter. Erythrocytes, 20,000–30,000/0·5 ml. were suspended in Prymnesium growth medium. Haematocrit values were estimated at 20° and readings in the Coulter particle counter were made at 3°, 20°, and 30°. Threshold readings at 50 % of the distribution curve of the population were made and the Counter was set as shown in Fig. 1. Erythrocytes: ● = human, ▲ = rabbit, ■ = mouse.

measurements made under standard temperature conditions. In Fig. 2, threshold values of 50 % of the population of erythrocytes were plotted against the volume of these cells as measured by a micro-haematocrit method (Strumia, Sample & Hart, 1954). The results obtained, like those described by Peacock, Williams & Mengoli (1960) and by Grant, Britton & Kurtz (1960), showed that a linear relationship existed between the threshold values and the volume measurement arrived at by the haematocrit method.

For the determination of the lytic activity of weak electrolytes an additional method was used, based on the estimation of the minimal effective dose (MED) of various agents capable of inducing lysis in Prymnesium populations. The test was carried out in test tubes containing 4·5 ml. of Prymnesium culture. The pH value of the medium was adjusted by adding dilute NaOH or HCl and 0·5 ml. of glycine + NaOH buffer (Sørensen; pH 6·0–9·3) or of sodium acetate + acetic acid buffer (Michaelis; pH 5·0–6·5). The cultures were kept at standard temperatures in continuous white daylight and microscopic observations made at different times. The MED value expresses the minimal concentration of weak electrolytes which induces total lysis of the Prymnesium population within 24 hr.

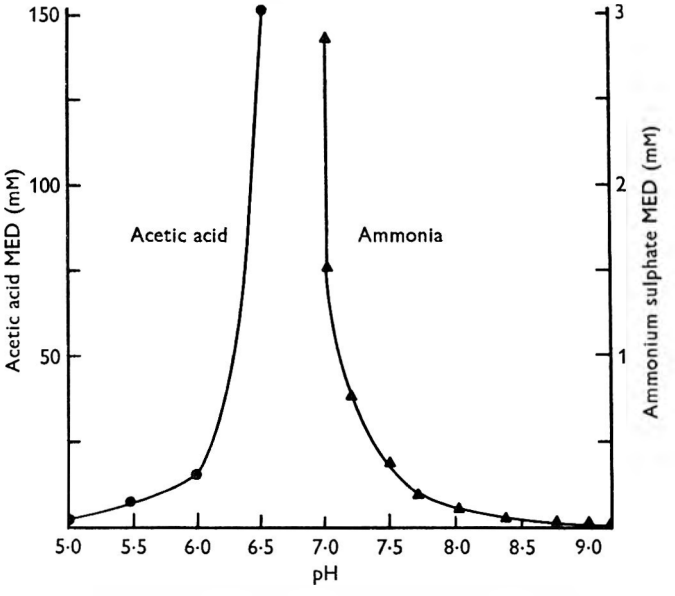


Fig. 3. The effect of pH value on lysis of Prymnesium by ammonia and acetic acid. Organisms from a 4-day culture were suspended in 4·5 ml. of Prymnesium growth medium; the pH value was adjusted as described under Methods; 0·5 ml. vols. of different amounts of ammonium sulphate or of sodium acetate + acetic acid buffer were added. The minimal effective dose (MED) was determined after 24 hr. at 25°.

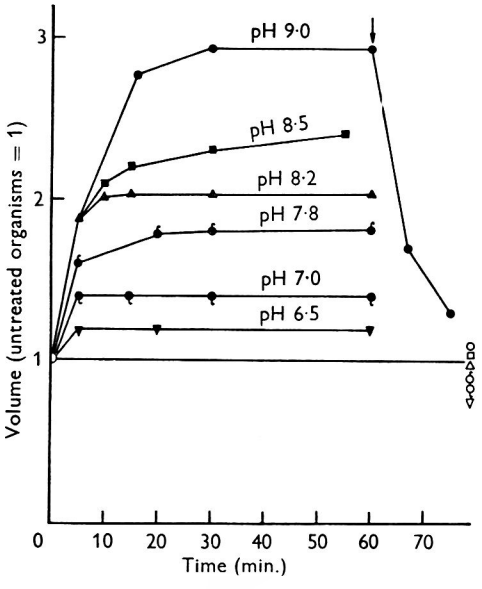
RESULTS

Effect of pH value on the lysis of Prymnesium by ammonia and by acetic acid

Observations with the microscope have shown that the nature of the lethal activity of ammonium sulphate on Prymnesium discovered by Reich & Aschner (1947) was a lysis which followed swelling of the organisms (Shilo & Shilo, 1961). It was further shown that the lytic activity of ammonia was dependent on the pH value of the milieu (Shilo & Shilo, 1953, 1955). In the present work various weak acids (acetic, citric, oxalic, and tartaric acids) were tested for their lytic activity on Prymnesium. Only acetic acid was found to have marked lytic activity on Prymnesium and, as with ammonia, this activity was pH-dependent. Figure 3 summarizes results of the effect of the pH value (in the range pH 5·0–9·0) on the lytic activities

of ammonium sulphate and of acetic acid. It can be seen that the lytic activity of ammonia increased with increasing pH value while the activity of acetic acid was greater the smaller the pH value. From these findings it was concluded that the undissociated acetic acid molecule rather than the acetate ion was the active compound in the lysis of Prymnesium, a condition like that suggested for lysis of Prymnesium by ammonia (Shilo & Shilo, 1953, 1961).

Direct measurements of the kinetics of swelling of Prymnesium induced by ammonia at various pH values were made; a typical experiment is summarized in Fig. 4. It was found that with increasing pH value of the suspension medium from pH 6·5 to 9·0 the rate of swelling increased and the final volumes of the swollen organisms were greater the greater the pH value. Upon change from pH 9·0 to 6·5,



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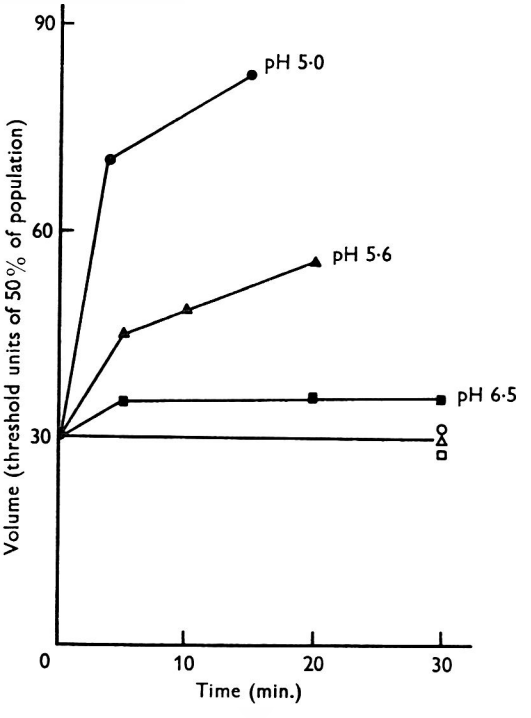

Fig. 5

Fig. 4. Effect of pH value of medium on the swelling of Prymnesium induced by ammonia. Organisms of a 4-day culture were suspended in Prymnesium growth medium which was divided into samples adjusted to different pH values and kept at 30°. Cell volumes were determined at different times by the Coulter particle counter, set as described in Fig. 1. The results are given in relative volume units; untreated organisms = 1. Solid symbols: suspensions containing ammonium sulphate 0·1 % (w/v). Open symbols: cell suspensions, at corresponding pH values without ammonium sulphate. ↓ = change of pH from pH 9·0 to 6·5.

Fig. 5. Effect of pH value on swelling of Prymnesium by acetic acid. Organisms of a 4-day culture were suspended in Prymnesium growth medium. The suspension was divided into samples adjusted to different pH values and kept at 30°. Acetic acid + sodium acetate buffer of appropriate pH value was added to a final concentration of 0·01 M. Solid symbols = suspensions containing acetic acid + sodium acetate (0·01 M). Open symbols = suspensions at corresponding pH values without acetic acid.

the volume of the organisms already swollen by contact with ammonium sulphate at pH 9 quickly returned to a volume near that of untreated organisms. In Fig. 5 the effect of the pH value of the suspension medium on the swelling of Prymnesium organisms in contact with acetic acid is shown. Spheres of polystyrene (Dow Chemical Company, Midland, Michigan) 8–16 μ diam., spores of the gastromycete *Rhizopogon luteolus* or pollen of grassweed (Coulter Electronics, Chicago) suspended in the Prymnesium growth medium served as controls in these experiments. When measured in the Coulter particle counter none of these particles showed any increase in volume after addition of ammonia or acetic acid at pH and temperature conditions optimal for the swelling of Prymnesium.

If lysis of Prymnesium depends on differences between external and intracellular pH values, as suggested by Jacobs (1940) for mammalian erythrocytes, no lysis should occur with ammonia or acetic acid in conditions where the pH value of the medium approaches that of the interior of the Prymnesium organism. This should give a method for measuring the internal pH value of the organism as a whole, or of different internal regions, if such exist. Indirect measurements of internal pH values, based on interior accumulation of weak electrolytes, have been made in yeasts with acetic acid (Conway & Downey, 1950), in Valonia with H_2S (Osterhout, 1925) and by following the uptake of ammonia in Valonia (Cooper & Osterhout, 1930), in Nitella (Irwin, 1925*a*) and in Halicystis (Blinks, 1933). To determine the internal pH value of Prymnesium, 'the point of no lysis' was approached from the alkaline side with ammonia and from the acid side with acetic acid. With both compounds no lysis occurred in the region between pH 6·5 and 7·0 (Table 1) thus suggesting that the internal pH value of Prymnesium is in this pH range.

Table 1. *Lysis of Prymnesium by ammonia and acetate at different pH values*

Organisms grown for 4 days in Prymnesium growth medium were used. The pH value of the suspending medium was adjusted as described under Methods. Lysis within 4 hr. after addition of weak base or weak acid at 25° was observed microscopically in the phase contrast microscope (Reichert Zetopan). Degree of lysis was recorded as: + + +, 100 %; + +, 50 %; +, 25 %; −, 0 %.

	pH value of suspension medium					
	5·5	6·0	6·5	7·0	7·5	8·0
	Degree of lysis					
Acetic acid + acetate 0·01 M	+ + +	+ +	+	−	−	−
Ammonium sulphate 0·01 M	−	−	−	+	+ +	+ + +

The effect of age of Prymnesium on sensitivity to swelling by ammonia

The effect of the age of Prymnesium organisms on their sensitivity to swelling by ammonia is shown in Fig. 6. Organisms from young cultures (2–4 days) in the early logarithmic phase of growth were compared with organisms taken after 13 and 30 days of incubation. On contact with ammonia the young organisms swelled much more rapidly than the organisms from the older cultures. The young organisms

reached their final volume within 1–5 min., swelling from 250 μ^3 to about 500–600 μ^3, while the older organisms continued to swell slowly up to 60 min. and reached smaller final volumes.

The effect of temperature on lysis of Prymnesium by ammonia

The effect of temperature in the range of 0°–30° on the lysis of Prymnesium by ammonia was tested; the results are given in Fig. 7. The rate of swelling as well as the final volumes of the organisms increased with increasing temperature. These results are in agreement with our earlier findings on the correlation between temperature and lysis by ammonia as expressed by the minimal effective dose of ammonium sulphate in cultures of Prymnesium and the temperature dependence of its algicidal activity against Prymnesium blooms in fishponds (Shilo & Shilo, 1953).

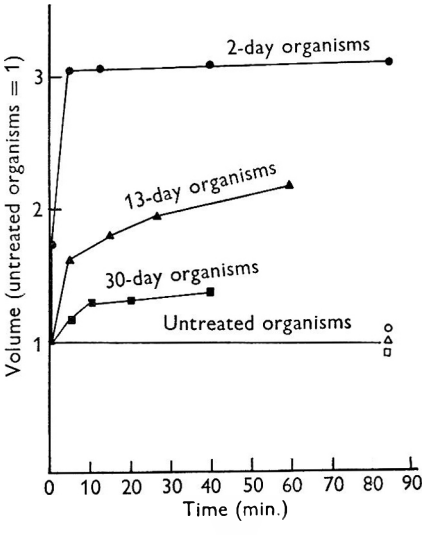

Fig. 6

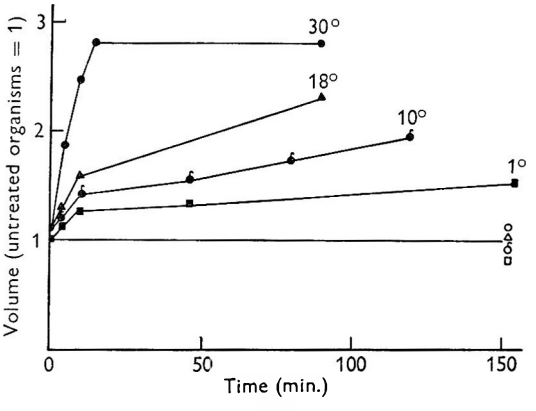

Fig. 7

Fig. 6. The effect of age of Prymnesium organisms on the kinetics of swelling by ammonia. Organisms were suspended in Prymnesium growth medium at pH 9·0. Readings were taken at 30° in the Coulter particle counter set as described in Fig. 1. Volume is given in relative units; untreated organisms = 1. Solid symbols: suspensions containing ammonium sulphate 0·1 % (w/v). Open symbols: suspensions of corresponding age without ammonium sulphate.

Fig. 7. The effect of temperature on the course of swelling of Prymnesium organisms induced by ammonia. Organisms of a 4-day culture were suspended in Prymnesium growth medium and kept at different temperatures at pH 9·0. Readings were taken in Coulter particle counter (set at gain switch, 7; gain trim, 3; aperture current setting, 4). Volumes as relative units; untreated organisms = 1. Solid symbols: suspensions containing ammonium sulphate 0·1 % (w/v). Open symbols: suspensions of corresponding temperatures without ammonium sulphate.

The effect of osmotic pressure of the milieu on lysis of Prymnesium by ammonia and acetic acid

Different weak electrolytes were found to act alike in causing lysis of Prymnesium, this effect resembled the ammonia-induced lysis of human erythrocytes described by Jacobs (1940). Attempts were made to see whether an osmotic mechanism underlies the lysis of Prymnesium induced by weak electrolytes. The lytic activity of ammonia and acetic acid on Prymnesium at different osmotic pressures of the suspension medium was tested. Figure 8 summarizes results testing different salt concentrations on the lytic activity of ammonium sulphate. It can be seen that the minimal amount of ammonia which caused lysis was a function of the external osmotic pressure. For the marine strain of Prymnesium and for the strain isolated from brackish fishponds, the lytic activity of ammonia salts was found to decline

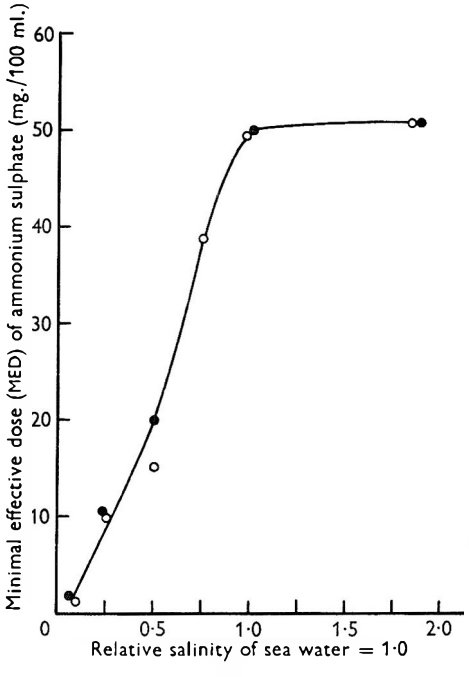

Fig. 8

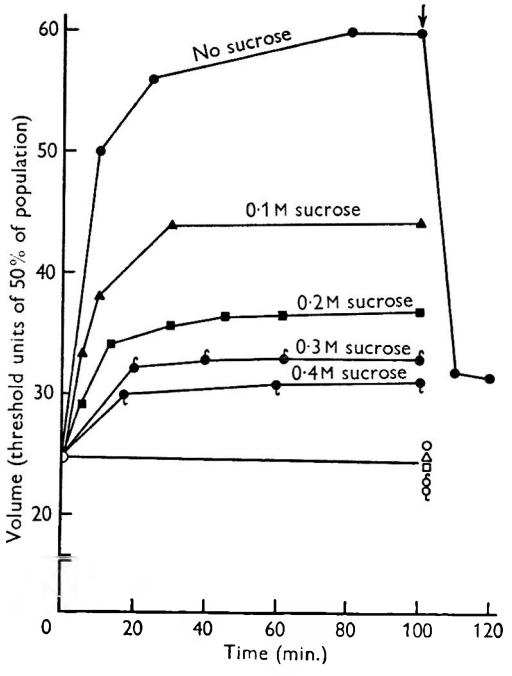

Fig. 9

Fig. 8. Effect of osmotic pressure of the suspending medium on sensitivity of Prymnesium to lysis by ammonium sulphate. Organisms of 4-day cultures of the two Prymnesium strains were suspended in Droop medium of different salinities by altering sea water and sodium chloride content. A relative salinity of 2·0 was obtained by using concentrated sea water. Minimal effective dose (MED) was determined at pH 8·8 at 25° as described under Methods. ● = Prymnesium isolated from sea water (Droop's strain); ○ = Prymnesium isolated from brackish water fishponds (Reich's strain).

Fig. 9. The effect of sucrose on the course of swelling of Prymnesium organisms by ammonia. Organisms of 2-day cultures were suspended in Prymnesium growth medium and kept at 30°, pH 9·0, with different amounts of sucrose. Volume was estimated in the Coulter counter, set at gain switch, 7; gain trim, 3; aperture current setting, 4. Solid symbols = suspensions containing 0·1 % (w/v) ammonium sulphate. Open symbols = suspensions of corresponding sucrose concentration without ammonium sulphate. ↓ = addition of sucrose (0·4 M).

with increasing osmotic pressure of the suspension medium. Change of external pressure by addition of sucrose (0·1–0·4 M) to the suspension medium caused a marked delay in swelling and lysis of Prymnesium by ammonia or acetic acid. Figure 9 shows the effect of different sucrose concentrations on the kinetics of swelling of Prymnesium organisms by ammonia as measured in the Coulter counter.

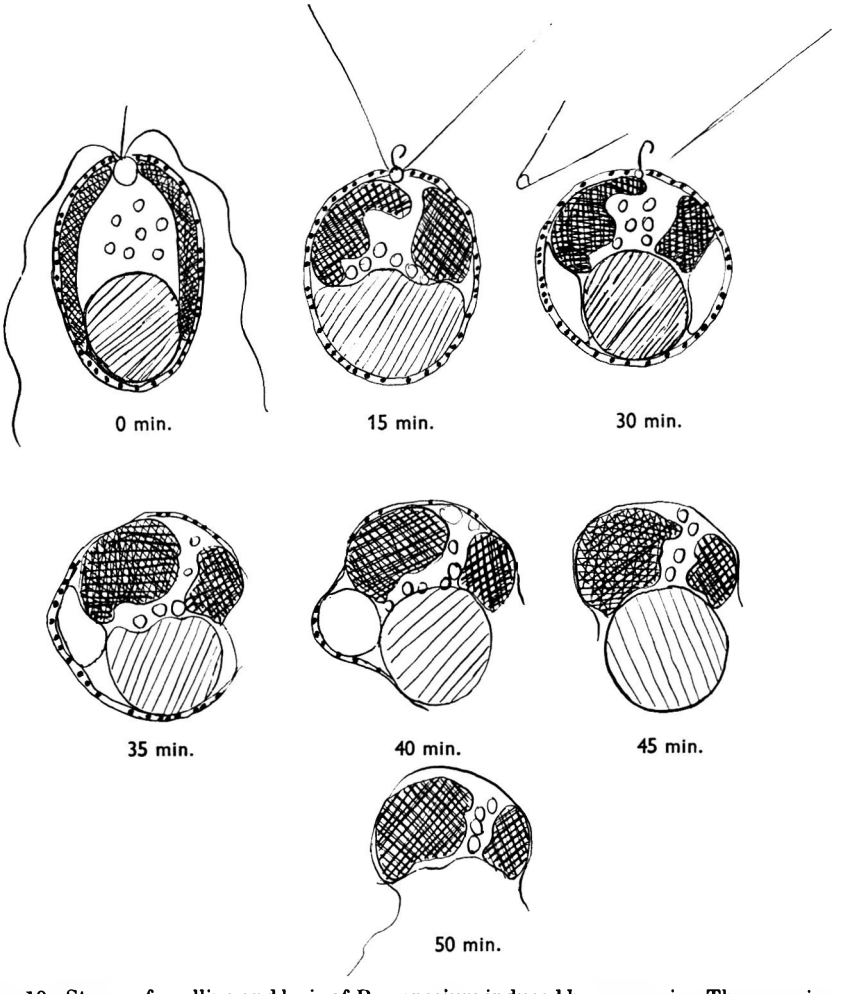

Fig. 10. Stages of swelling and lysis of Prymnesium induced by ammonia. The organisms were grown in Prymnesium growth medium for 4 days. The stages of swelling and lysis of a typical Prymnesium organism in contact with 0·1 % (w/v) ammonium sulphate at pH 9·0 at 25° were drawn with the aid of a camera lucida under the phase contrast microscope (Reichert Zetopan). Enlargement × 1250.

Sucrose at 0·4 M almost completely inhibited swelling of the organisms; with lower concentrations of sucrose partial suppression of swelling was observed. Addition of sucrose to 0·4 M to organisms already swollen by ammonia to their maximal size in absence of sucrose led to immediate shrinkage of the organisms until the volume attained by Prymnesium organisms in similar sucrose concentrations was reached.

The morphological changes in swelling and lysis of Prymnesium induced by ammonia and by acetic acid

The sequence of morphological changes leading to lysis by weak electrolytes was observed under the phase-contrast microscope. Prymnesium organisms swell considerably when put into ammonia or acetic acid at appropriate pH values and temperatures, and they are very suited for the study of visual changes of internal structure. Furthermore, the rupture of the organism causes the liberation of internal organelles into the medium and these can be observed under various experimental conditions (Shilo, 1961).

The morphological changes leading to lysis of Prymnesium by ammonia are shown in camera lucida drawings (Fig. 10) and in photomicrographs (Pl. 1). They show the sequence of events in single organisms undergoing swelling and lysis. When ammonia was added at appropriate pH values and temperatures the organisms began to swell because of the enlargement of two lateral vacuoles, thus causing the usually elongated organism to take a swollen ball-like form. At this stage the flagella broke off, the contractile vacuole at the anterior part of the organism stopped its rhythmical beating and the elongated chromatophores were pushed to the anterior part of the organism. The organism continued to swell. This was seen in the phase microscope by noting the increase in distance between the granules in the envelope. Upon rupture of the envelope the two lateral vacuoles became spherical and continued to swell until they burst. With lysis induced by acetic acid the sequence of morphological changes seen in the phase contrast microscope was different from that seen during lysis by ammonia. In lysis with acetic acid, like lysis in hypotonic conditions (Pl. 1), the organisms swelled because of increase in volume of the large posterior vacuole and finally lysed. The lateral vacuoles which were the site of swelling in ammonia-induced lysis did not become visible at all during the process of lysis with acetic acid.

DISCUSSION

The pH-dependence of lysis of Prymnesium by weak electrolytes may be explained by the fact that the organism is permeable to the undissociated electrolyte but not to the ion. This is in agreement with results on the penetration of weak electrolytes found with other biological systems (Irwin, 1925*a*, *b*; Osterhout, 1925; Cooper & Osterhout, 1930; Collander, Turpeinen & Fabritius, 1931; Jacobs & Parpart, 1938; Conway & Downey, 1950; MacMillan, 1956).

The degree of concentration of weak electrolyte inside the organism is suggested to be dependent upon the difference existing between the interior and exterior pH values. These relationships were expressed for mammalian erythrocytes by Jacobs (1940), based on the Hasselbach–Henderson equation for the pH values of aqueous solutions of weak electrolytes, as follows:

$$C = \frac{1+10^{pH_o-pK}}{1+10^{pH_i-pK}} \cdot C_o$$

where C = total concentrations of all forms of NH_3;
o = outside the erythrocyte; i = inside the erythrocyte.

This equation allowed the calculation of the total concentration of all forms of the weak electrolyte within the cell at different pH values, different strengths of electrolyte, and different electrolytes.

The results with Prymnesium showed that there was a general similarity between the behaviour of erythrocytes and the phytoflagellate as to the dependence of the lytic activity of ammonia on the pH value of the medium (Shilo & Shilo, 1955, 1961). Table 2 shows that the experimental values for the lytic activity of ammonia at different external pH values, as expressed by the minimal effective dose (MED) of the weak electrolyte, were near the values calculated by the equation proposed by Jacobs.

Table 2. *The lytic activity of ammonium sulphate on Prymnesium at different pH values*

pH value	Concentration of total intracellular ammonia calculated concentration factor C_i/C_o*	Lytic activity of ammonium sulphate. Experimental value†
7·0	1	1
8·0	10	9
9·0	64	56

* Intracellular concentration factor C_i/C_o was calculated on the basis of the equation of Jacobs (1940) assuming internal pH 7·0 for Prymnesium.

† The experimental value of lytic activity is expressed as a function of the reciprocal of minimal effective dose of ammonium sulphate on Prymnesium. For comparison, the minimal effective dose of $(NH_4)_2SO_4$ (38 mg. %) at external pH 7·0 has been taken as 1.

The findings of the present work suggest that an osmotic mechanism underlies the lytic activity of weak electrolytes upon Prymnesium. The weak electrolytes accumulate in the organism until the internal osmotic pressure is greater than the pressure which the envelope of the organism can resist. The osmotic nature of the lysis of Prymnesium induced by weak electrolytes is further suggested by its dependence on the osmotic pressure of the suspending medium. As might be expected with an osmotic mechanism of lysis, the organisms were more sensitive to lysis by weak electrolyte the smaller the osmotic pressure of the suspension medium. That acetic acid and ammonia lyse Prymnesium is consonant with the idea that lysis is due to entry of weak electrolyte which causes an osmotic imbalance, rather than that these compounds have some other toxic effect.

The morphological changes of Prymnesium organisms, during lysis caused by a weak acid or a weak base, showed that the increase in size of different regions of the organism was responsible for the swelling which leads to rupture. These findings might be explained by a difference in the pH value of different regions. It would follow that the lateral vacuoles responsible for lysis by ammonia must have a pH value smaller than that of the posterior vacuole responsible for lysis by acetic acid. These vacuoles of Prymnesium seem to behave like different compartments each surrounded by a semipermeable membrane.

The drastic increase of lytic activity of ammonia towards Prymnesium with increase in temperature (range 0°–30°) indicates that the factors which influence the concentration of the weak electrolyte inside the organism must be much more temperature-sensitive than would be expected on the basis of penetration by

diffusion alone. Irwin (1925*b*) showed that penetration of the weak electrolyte brilliant cresyl blue in undissociated form into Nitella had a high-temperature coefficient.

The close agreement of our experimental findings with the theoretical predictions of Jacobs makes it likely that a concentration mechanism dependent on pH differences is operative. However, it may perhaps be supposed that the functioning of a temperature-sensitive metabolic process can affect the rate of swelling and final volume of an organism. Such a process which might affect the lysis of Prymnesium by a weak electrolyte might be that of an internal pH stabilization of different intracellular regions.

Ammonia has found wide application as an effective agent in controlling mass development of Prymnesium in brackish-water fishponds, thus preventing extensive fish mortality from the toxin produced by Prymnesium. On the basis of the proposed osmotic mechanism better understanding of the reasons which underlie the algicidal activity of ammonia in fishponds may come from the proposed osmotic mechanism. Resistance of Prymnesium to the lytic activity of ammonia salts often found in ponds was shown to be mainly due to small pH values, low temperatures, or the relatively high salinity of the pond water. The results described may also explain the outstanding sensitivity of Prymnesium to lysis by ammonia or acetic acid in comparison with other micro-organisms. Prymnesium until recently was the only known nucleated organism which in relatively low concentrations of these weak electrolytes undergoes lysis. *Prymnesium parvum* has a normal habitat of high salinity, i.e. it can develop in salt concentrations of 6 %, but can also thrive in a milieu containing only 0·1 % as in brackish fishponds. This is about the lowest degree of salinity in which this phytoflagellate is able to survive. Under such conditions even a slight increase in the internal osmotic pressure as may be caused by accumulation of ammonia or acetic acid at certain pH values and temperatures could cause osmotic imbalance and lead to lysis.

Other micro-organisms such as the flagellate *Dunaliella salina* Teodor and *Syracosphaera carterae* and also certain luminous marine bacteria were found to swell and lyse when brought in contact with ammonia under conditions of high external pH and when the osmotic pressure of the surrounding medium approached the lower limit for viability of these organisms. These findings indicate that lysis of microbial cells by weak electrolytes may, in fact, be a widespread and general phenomenon, provided the cells are already under conditions of severe osmotic stress.

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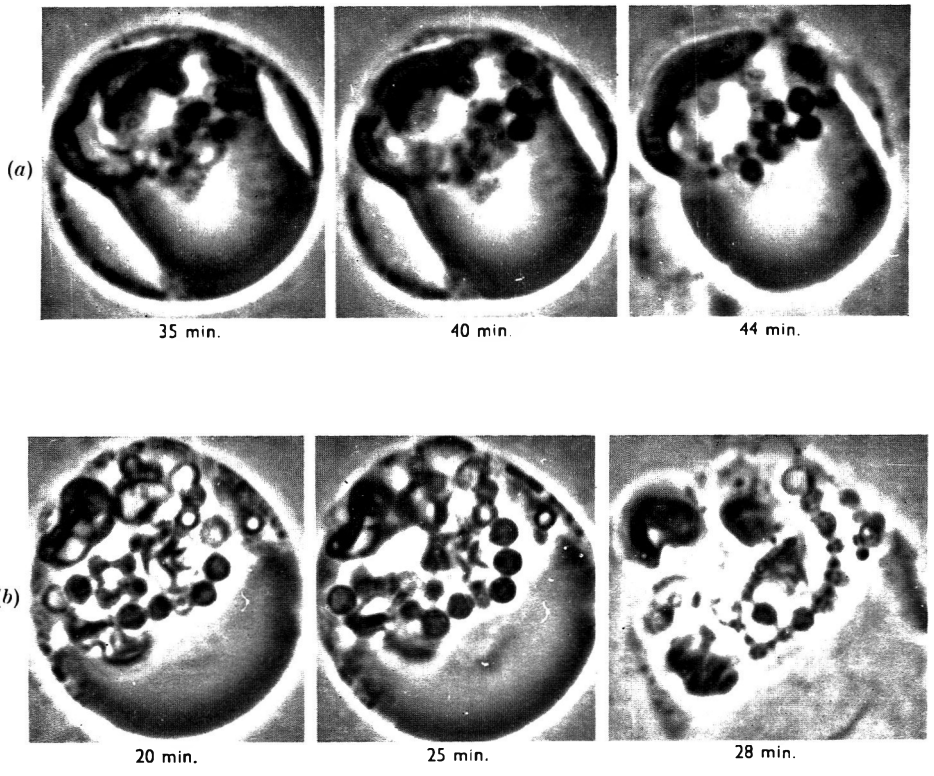
EXPLANATION OF PLATE 1

Photomicrographs of the swelling and lysis of typical single Prymnesium organisms followed under the phase contrast (Reichert Zetopan) oil immersion. ×1250. Cells were suspended in Prymnesium growth medium containing (*a*) 0·1% (w/v) ammonium sulphate at pH 8·5 at 25°; (*b*) 0·02 M-acetate at pH 5·0 at 25°; (*c*) cells suspended in distilled water.


(a)
35 min.
40 min.
44 min.
(b)
20 min.
25 min.
28 min.
(c)
5 min

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Superinfection of Lysogenic Strains of *Salmonella typhimurium* Q1: Prophage Substitution and Double Lysogenization

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SUMMARY

Strains of *Salmonella typhimurium* Q1, lysogenized with type A phages, were superinfected with the heterologous free phages of the same group. This produced lysis (productive or vegetative development) and prophage change (either prophage substitution or double lysogenization) in a constant pattern. Prophage change was frequently detected when lysis was absent. Certain of the phages were aggressive, producing active lysis and prophage change in many of the heterologous lysogenic strains, others were intermediate and some were non-aggressive. In general, aggressive phages in prophage form conferred a good degree of immunity on the host bacterium, while non-aggressive phages did not: but to this rule there were exceptions. In most cases, immunity to lysis and immunity to prophage change ran an approximately parallel course, but again there were exceptions. Some strains, with certain superinfections, were immune to lysis but not to prophage change, while others showed greater resistance to prophage change than to lysis. The reaction to superinfection split the series into two groups. Superinfection of a lysogenic organism of either group with phage of the same group produced—if there was any prophage change—prophage substitution: superinfection of a lysogenic organism of one group with phage of the other group produced double lysogenization. Each group of phages had therefore its own site of attachment to the bacterial chromosome. Immunity appeared to be due neither to defective adsorption nor to steric interference, but to repressors with specific characters which varied from strain to strain.

INTRODUCTION

Lysogenic bacteria are generally (but not invariably) immune to the temperate phage which they produce, and in some cases, but in a more limited way, to related temperate phages of the same group. The cross-immunity test, used to differentiate and identify twelve of the temperate Type A phages found in *Salmonella typhimurium*, is based on the varying degrees of resistance found in bacteria lysogenized by these phages (Boyd & Bidwell, 1957). Absence of resistance is shown by plaque formation resulting from the lytic action of the superinfecting phage. In addition to producing lysis, the superinfecting phage may bring about changes in the prophage content of some of the bacteria which escape lysis (Boyd, 1956). Briefly, the superinfecting phage may either evict and replace the prophage of the non-immune lysogenic bacteria—'prophage substitution'; or it may itself become prophage without disturbing the resident prophage, thus causing double lysogeniza-

tion in the bacterium. In certain cases 'cure' has been noted, where the superinfecting phage has evicted the resident prophage, but has failed to establish itself as a replacement. These phenomena have now been investigated in some detail, and in this paper an account is given of the more important findings.

METHODS

The cultures and bacteriophages used in these experiments, and also the culture media and fundamental techniques, have been fully described in previous papers (Boyd, 1950; Boyd & Bidwell, 1957, 1959, 1961). The terms adopted to designate cultures and phages are those in common use. *Salmonella typhimurium* Q1 is referred to as Q1: phage A1*a* is simply A1*a*. Q1 carrying prophage A1*a* (i.e. lysogenic) is Q1(A1*a*). The expression 'prophage change' is used to indicate either prophage substitution or double lysogenization. In quoting phage:bacteria ratios the phage is always shown first: thus a 1:10 ratio indicates 1 phage particle to 10 bacteria.

The first method used to establish the pattern of prophage change was based on the cross-immunity test already described (Boyd & Bidwell, 1957). A loopful of the superinfecting phage—at least 10^{10} particles/ml.—was placed on a lawn of the lysogenic bacteria, and the plate incubated overnight at 37°. Whether a patch formed or not (a reaction indicating the occurrence or otherwise of lytic action of the superinfecting phage on the lysogenic bacteria), a small quantity of the material from the centre of the spot where the phage had been placed on the lawn was picked up with a sterile platinum needle and rubbed into 1 ml. of sterile broth in a test tube. A drop of this suspension was then spread on nutrient agar and incubated overnight. Accurately gauged quantities produced a plate peppered with discrete colonies. When the superinfecting phage had a lytic action on the lysogenic bacterium, some of these colonies were phage-contaminated, and presented the characters described in an earlier paper (Boyd, 1951): others were normal in appearance. Twenty colonies of normal appearance were selected, cultured in broth, and replated to confirm the absence of surface phage contamination. Those which showed evidence of contamination were discarded. Thereafter the colonies were subjected to various tests to determine their prophage content. As there were in all 132 different combinations of phage and lysogenic organisms, it is impracticable to give details of the exact steps in each identification. In most cases a modified cross-immunity test provided a simple and reliable means of determining if any prophage change had taken place. In a few it was found helpful to propagate the phage on *Salmonella typhimurium* strain 1404, which supports the growth of the A1 group, A3, and A4, but not the A2 group. This selective propagation was of particular value in some of the double lysogenizations, where the separation of the two phages occasionally presented considerable difficulty. Where necessary the identity of a phage was confirmed by a full-scale cross-immunity test. Control tests were made which showed that external phage contamination was not a source of error. Where the superinfecting phage lysed and so 'marked' the colony, this danger did not arise, as it was an easy matter to select uninfected colonies from a plate. In the absence of such lytic action, there was no marking of contaminated colonies. However, as the absence of lysis showed that there was no active propaga-

tion of the superinfecting phage, the chance of any residual free phage remaining in cultures made from individual colonies, each the product of a single organism, was negligible, especially when this process was twice repeated. Selected doubly lysogenized strains, repeatedly subcultured, maintained their characters indefinitely. Further, as will be seen later, the fact that either prophage substitution or double lysogenization occurred in a definite and predictable pattern afforded convincing confirmation of the reliability of this finding.

Table 1. *Method* 1: *prophage change*

The denominator shows the number of colonies examined, the numerator the number in which prophage change—either substitution or double lysogenization—occurred.

	Salmonella typhimurium Q1 carrying prophage											
Phage type	A1*a*	A1*b*	A1*c*	A1*d*	A2*a*	A2*b*	A2*f*	A3	A4	A2*c*	A2*d*	A2*e*
A1*a*	·	13/20	0/20	10/20	7/20	0/20	0/20	0/20	0/20	1/20	4/20	3/40
A1*b*	17/20	·	1/20	10/20	8/50	29/109	17/49	4/40	0/20	7/24	2/20	4/20
A1*c*	15/19	16/20	·	11/20	6/20	10/18	14/20	4/40	0/20	1/20	2/20	16/20
A1*d*	12/20	17/20	0/20	·	0/20	0/20	10/40	10/10	0/20	1/20	7/20	20/20
A2*a*	2/18	15/20	0/20	0/20	·	9/20	13/20	8/40	0/20	9/20	5/20	16/20
A2*b*	13/19	43/57	1/44	14/20	11/20	·	14/20	7/30	2/60	20/32	2/20	5/18
A2$_f^f$	0/60	5/20	0/20	0/20	1/20	0/20	·	0/20	0/20	5/50	2/40	3/40
A3	0/40	5/20	0/20	2/20	4/20	0/20	0/20	·	0/40	5/20	2/20	2/20
A4	31/40	10/20	0/20	0/20	0/20	1/20	13/39	0/40	·	5/20	2/20	10/20
A2*c*	28/44	44/44	0/60	1/20	4/20	12/36	15/40	19/20	0/20	·	4/20	10/20
A2*d*	13/20	3/20	2/20	3/19	3/20	7/20	32/60	20/20	0/20	1/20	·	11/20
A2*e*	15/20	10/20	16/20	24/40	13/20	3/24	13/40	35/40	23/30	4/20	15/20	·

In Table 1 figures are given with the number of colonies showing prophage change as the numerator and the number examined as the denominator. These give only a rough indication of the degree of reaction, not an exact quantitative measurement. Nevertheless, when repeated tests were made the results as a rule showed no gross variation, and the general picture which the figures present may be regarded as of some value, particularly at the lower limits. This simple technique will be referred to as method 1. A more precise quantitative estimate of the degree of prophage substitution or double lysogenization and of the associated productive infection (vegetative development or bursts) was obtained by means of two different techniques.

In certain cases (e.g. Q1 (A1*a*) superinfected with A1*b*) a modification of Levine's (1957) method was used. *Salmonella typhimurium* T gal$^-$, kindly given to us by Dr Prell, is sensitive to A1*a*, and it was therefore possible to prepare a lysogenic strain, T gal$^-$ (A1*a*), which was immune to A1*a*. Q1 is gal$^+$, and so outgrew T gal$^-$ (A1*a*) when plated as a dilute suspension on a lawn of this organism on galactose agar. A1*b* produced plaques freely on T gal$^-$ (A1*a*). Thus on a lawn of T gal$^-$ (A1*a*), bursts liberating A1*b* produced plaques best seen after incubation for 24 hr. at 37°, while colonies of Q1 (A1*b*) could be distinguished by the halo which surrounded them when, after 48 hr. incubation at 37°, the plates were kept for a further 24 hr. at room temperature. On the other hand Q1 (A1*a*) gave colonies which, by virtue of their more vigorous growth, stood out from the lawn of T gal$^-$ (A1*a*), but had no surrounding halo. In tests by this method, cultures of the lysogenic strain in the logarithmic phase of growth, diluted to a standard concentra-

tion (10^8 organisms/ml.) were exposed to graded concentrations of the superinfecting phage and placed in the water bath at 37° for 10 min. Thereafter each culture was diluted $10^{-5} \times 1/5$ (using, at an intermediate stage in the process of dilution, an antiphage serum to neutralize any non-adsorbed free phage), 0·5 ml. quantities were gently flooded on to a lawn of T gal$^-$ (A1*a*) on galactose agar, and plaques and colonies counted at the times indicated. A count of the viable bacteria in a control culture provided a figure from which could be calculated the percentage of bursts and of bacteria in which prophage substitution had occurred. Unfortunately this method, which will be designated method 2, has only a limited application. T gal$^-$ is resistant or partly resistant to several phages of the series, and of course double lysogenizations cannot be investigated in this way.

The preliminary steps of method 3 were similar to those in method 2, but the diluted suspension was plated directly on nutrient agar, and not on a lawn of lysogenized T gal$^-$ on galactose agar. Each colony which developed was tested for its prophage content. Bursts were calculated by the 'tube' technique (Boyd & Bidwell, 1959).

RESULTS

Preliminary investigations

Using method 1, each lysogenic strain was exposed to superinfection with the heterologous temperate phages of the series. It was at first assumed that the pattern of reactions would be similar to that of the cross-immunity test, and that prophage substitution or double infection would occur in parallel with the lysis which produced the patches or plaques in the cross-immunity test. This assumption

Table 2. *Superinfection with* A1*b*: *prophage change in the lysogenic bacteria of the series*

The figures give the percentage of bacteria in which prophage change occurred, estimated by method 3. Q1, exposed to A1*b* in a 1:1 ratio, showed approximately 45% lysogenization. A1*a* therefore afforded no protection: A1*c*, A4, A2*d* and A2*e* gave 100% immunity at this ratio: the others fell between these extremes.

	Exposed to A1*b* in 1:1 ratio												
	Salmonella typhimurium Q1 carrying prophage												
	Q1 control	A1*a*	A1*b*	A1*c*	A1*d*	A2*a*	A2*b*	A2*f*	A3	A4	A2*c*	A2*d*	A2e
Percentage of bacteria showing prophage change	45	45	—	0	34	31	21	41	2	0	2·5	0	0

proved to be wrong, for it was found that in some cases, where there was no detectable lysis, superinfection gave rise to prophage change. The results are shown graphically in Fig. 1, while in Table 1 figures are given which, subject to the reservations already made, give some indication of the degree of prophage change.

A more accurate estimation of the varying percentages of prophage change in the lysogenic strains superinfected with A1*b* at a 1:1 ratio is given in Table 2, and reference will be made in the text to results given by method 3 in other cases. A remarkable feature of these findings was the division of the twelve lysogenic strains into two groups differentiated by the occurrence of prophage substitution

or double lysogenization. This phenomenon will be fully discussed at a later stage. Apart from this one definite pattern, the picture is very complex. In an attempt to throw some light on its many puzzling aspects, numerous investigations were made, from which the following examples have been selected to illustrate the different types of reaction which have been encountered.

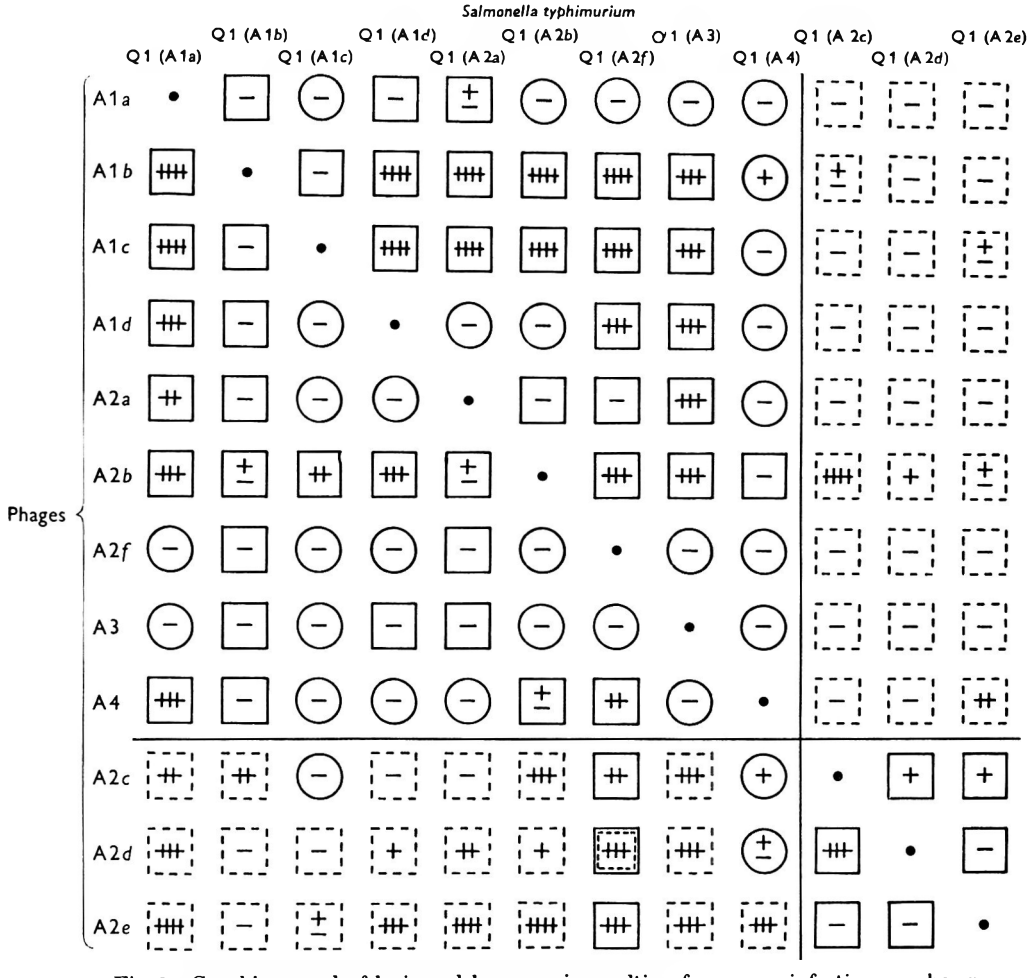

Fig. 1. Graphic record of lysis and lysogenesis resulting from superinfection, as shown by method 1. □, Prophage substitution; ⬚, double lysogenization; ○, no prophage change; ●, not tested; + + + + to −, degree of lysis at critical test concentration of phage (lowest concentration giving confluent lysis in control). Note: A2*d* against Q1 (A2*f*) 7/60 double lysogenization, 25/60 prophage substitution; A2*e* against Q1 (A1*d*), one colony found showing prophage substitution.

Prophage substitution

A1*a* ⇌ A1*b*. Prophage substitution is well illustrated by the action of A1*b* on Q1 (A1*a*). In terms of the cross-immunity test, this falls into the category of one-way reactions, as A1*a* does not produce visible lysis in Q1 (A1*b*), while the lytic action of A1*b* on Q1 (A1*a*) is well marked. Thus the plating efficiency of A1*b*

on Q1 (A1*a*) was virtually identical with its plating efficiency on non-lysogenic Q1, the plaque counts of the batch of A1*b* used in these tests being $1{\cdot}76 \times 10^{10}$/ml. on Q1, and $1{\cdot}7 \times 10^{10}$/ml. on Q1 (A1*a*). The plaques formed on the two strains appeared identical. A1*b* was equally well adsorbed on Q1 and Q1 (A1*a*) (Fig. 2). The opacity curves of growing cultures of Q1 and Q1 (A1*a*) exposed to different concentrations of A1*b* were alike, indicating corresponding degrees of vegetative development and

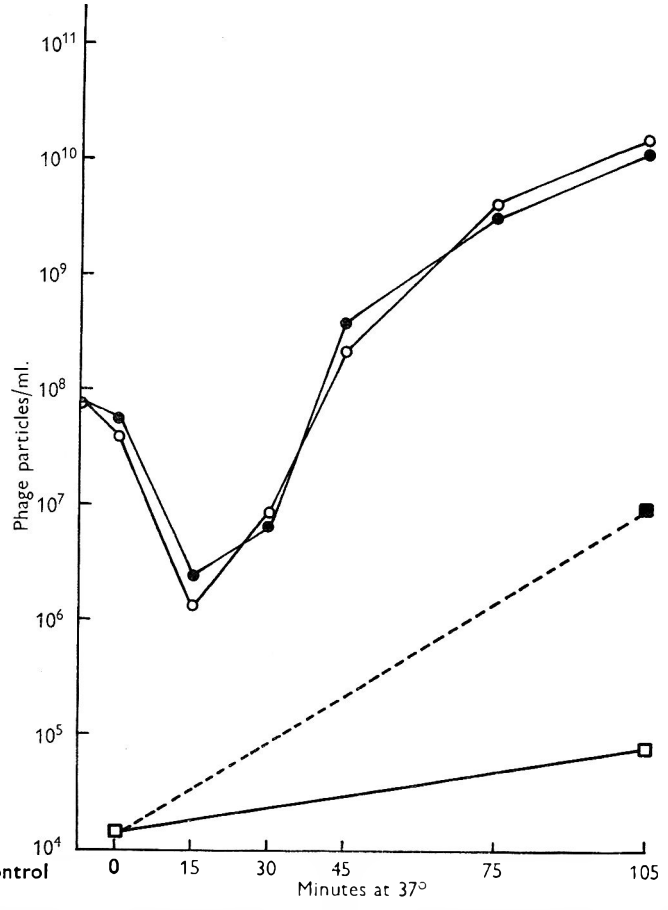

Fig. 2. Adsorption of A1*b* on Q1 and Q1 (A1*a*). Phage:bacteria ratio, in this and all other experiments unless specifically stated, was approximately 1:1. Incubation was in a water-bath at 37°. The pre-zero fall in phage titre was due to adsorption during the process of mixing before a specimen could be taken. Samples were removed at the intervals shown, immediately diluted in decimal steps to 10^{-6} to prevent further adsorption, and heated to 70° for 30 min. to kill the bacteria. Thereafter counted by standard method. All counts are plaque counts. Adsorption after 15 min. was obscured by phage production. There was no significant difference in the curves. There was some induction of Q1 (A1*a*) by A1*b*. ○, A1*b* on Q1; ●, A1*b* on Q1 (A1*a*); ■, A1*a* from induction of Q1 (A1*a*); □, A1*a* in control culture of Q1 (A1*a*).

presumably of lysogenization (Fig. 3). The adapted Levine technique was used to determine in some detail the reactions of Q1 (A1*a*) superinfected with A1*b*. The results are shown in comparison with results given by Q1 exposed to A1*b* (Table 3).

The similarity is striking, and indicates that prophage A1*a* confers no immunity against A1*b*. To control the results given by the Levine technique, cultures of Q1 (A1*a*), which had been exposed to A1*b* in a 1:1 ratio, were prepared in the same way as in the foregoing experiment, and cultured on plain nutrient agar. All colonies which developed on incubation were examined. The proportions which proved to be Q1 (A1*a*) and Q1 (A1*b*) were in good agreement with the figures given by the Levine technique: in addition, 1 % were found to be 'cured', i.e. did not contain either prophage A1*a* or prophage A1*b*.

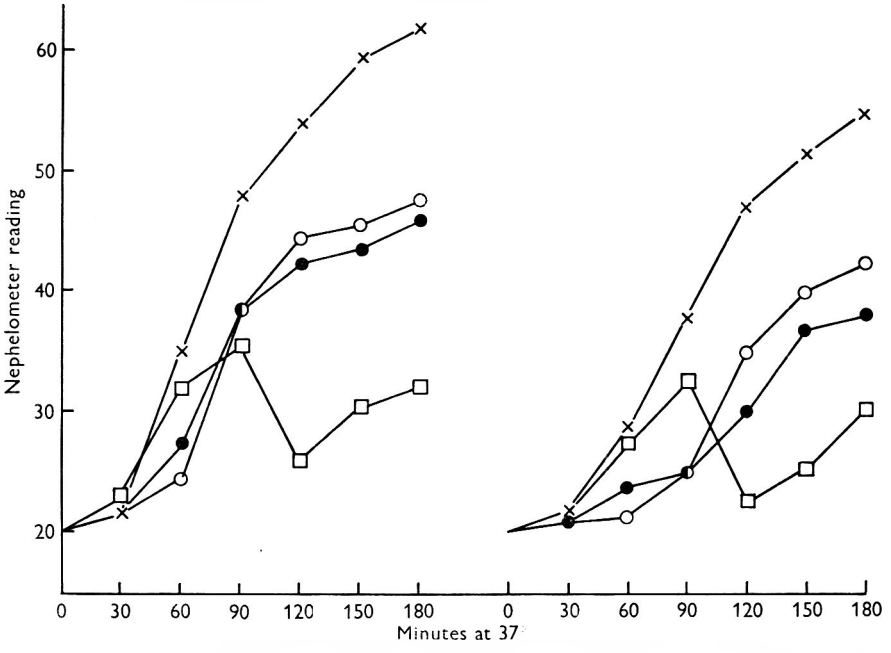

Fig. 3. Opacity curves of growing cultures of Q1 and Q1 (A1*a*) exposed to A1*b*. There was no significant difference. ×, Control; ○, 10:1 ratio; ●, 1:1 ratio; □, 0·1:1 ratio.

Table 3. *Comparison of Q1 infected and Q1 (A1a) superinfected with A1b*

The bacteria were throughout in a concentration of 10^8/ml.; phage was added in quantities giving the appropriate ratio. Lysogenics and bursts were estimated by the adapted Levine technique. The figures are the percentage of bacteria in which prophage change or bursts occurred.

Phage:bacteria ratio ...	0·1:1		0·31:1		1:1		3·1:1	
Phage A1*b* infecting or superinfecting	Q1	Q1 (A1*a*)	Q1	Q1 (A1*a*)	Q1	Q1 (A1*a*)	Q1	Q1 (A1*a*)
Lysogenized with A1*b*	7·3	7·2	16·3	18·5	43	45	64·5	70
Bursts	2·6	5·5	10	9·4	24·5	22·8	12	17

In contrast to this clear-cut picture, the reactions of Q1 (A1*b*) superinfected with A1*a* were less obvious. A1*a* did not form plaques on Q1 (A1*b*) and this absence of lytic action was confirmed both by the opacity curves which showed

no evidence of clearing at any of the phage:bacteria ratios, and by the adsorption test which showed that, although A1*a* was well adsorbed to Q1 (A1*b*), there was no subsequent increase in free phage resulting from vegetative development (Fig. 4). However, when examined by method 1, and when bacteria from the apparently negative 'patch' of A1*a* on Q1 (A1*b*) were plated, 13 out of 20 colonies selected

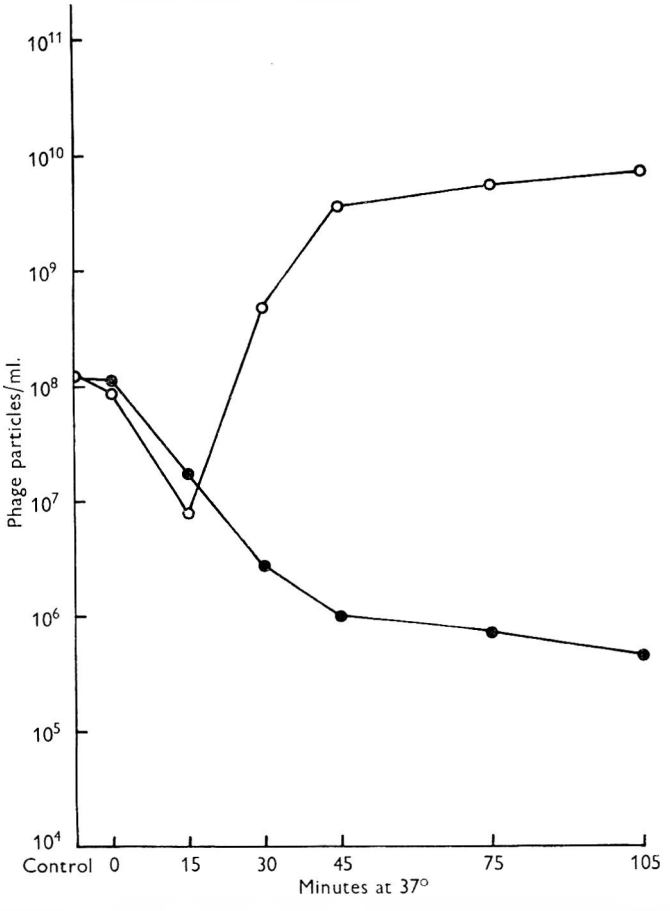

Fig. 4. Adsorption of A1*a* on Q1 and Q1 (A1*b*). Method as described for Fig. 2. On Q1, adsorption obscured after 15 min. by phage production, ○; on Q1 (A1*b*), adsorption without phage production, ●.

for further tests proved to be sensitive to the action of A1*b*, and gave the reactions of Q1 (A1*a*) in a full-scale cross-immunity test, while by method 3 at a 10:1 ratio prophage substitution took place in 1·7% of the superinfected bacteria. Thus despite the absence of detectable lysis an appreciable degree of prophage substitution had occurred, the 'aggressive' A1*b* prophage having been evicted and replaced by the apparently much less active A1*a*. No 'cures' occurred among the colonies tested.

*A*1b ⇌ *A*2b. These two phages have a more balanced reaction: each produces plaques—i.e. lysis—on the opposite lysogenic organism, though A1*b* is the more active of the two. On Q1 (A2*b*) the plating efficiency of A1*b* was more than halved,

the plaque count of the batch of phage used being $1{\cdot}1 \times 10^{10}$/ml. on Q1 and $4{\cdot}8 \times 10^{9}$/ml. on Q1 (A2*b*). The plaques on Q1 (A2*b*) had a granular centre, and showed gross variation in size. Adsorption of A1*b* on Q1 (A2*b*) and subsequent production of free phage showed a lag of 15 min. when compared with similar reactions between A1*b* and Q1 (Fig. 5). Opacity curves of cultures of Q1 (A2*b*) exposed to

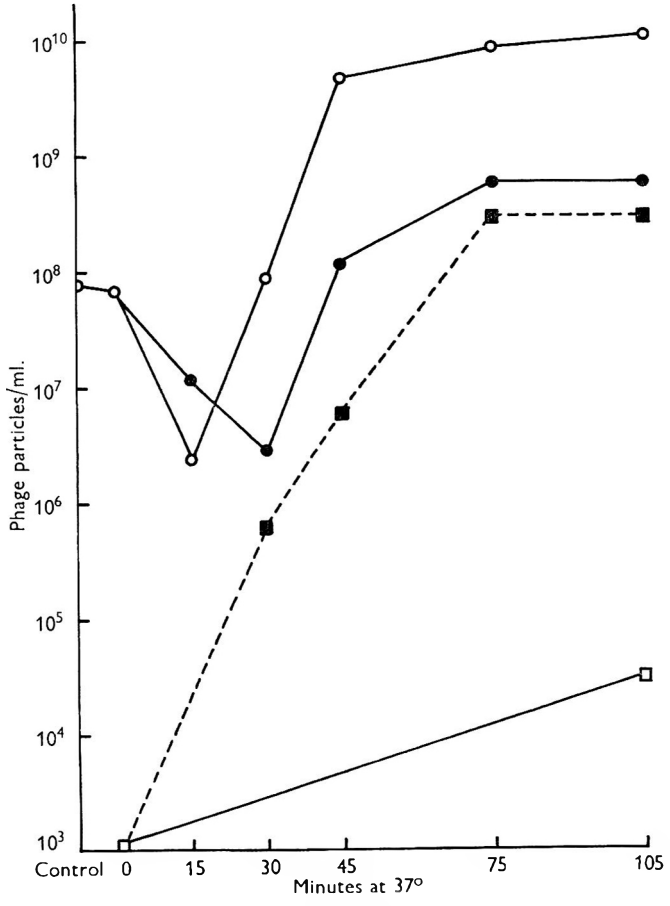

Fig. 5. Adsorption of A1*b* on Q1 and Q1 (A2*b*). Adsorption on Q1 (A2*b*) slower and phage production more limited. A1*b* both multiplied in and induced Q1 (A2*b*). ○, A1*b* adsorbed on Q1; ●, on Q1 (A2*b*); ■, A2*b* from induction of Q1 (A2*b*); □, A2*b* in control culture of Q1 (A2*b*).

A1*b* in a ratio of 1:10 showed well-marked clearing after 120 min. incubation, whereas in a control with Q1 the clearing began in 90 min.

Because of the low plating efficiency and in particular the atypical plaques, Levine's technique proved unsatisfactory, and the degree of conversion was determined by method 3, using a 1:1 ratio. 150 of the resulting colonies were tested for lysogenicity, and 30 proved to be Q1 (A1*b*), while 3 were cures, the remainder being unchanged Q1 (A2*b*). Calculated in terms of the count of viable bacteria in control cultures, this amounted to a conversion rate from Q1 (A2*b*) to Q1 (A1*b*)

of 14 %. Bursts were not calculated. The lysogenization of Q1 by A1*b* at the same phage:bacteria ratio is 45 %.

The batch of A2*b* used for the second half of this interaction, when titrated on Q1, had a plaque count of $2{\cdot}6 \times 10^{10}$/ml. On Q1 (A1*b*) the plating efficiency was greatly reduced, and the plaques were so shallow and small that an accurate count was impossible; from the results given by titration in decimal dilutions, it was estimated to be about 10^6/ml. Adsorption at a 1:1 ratio was of the usual order and was followed by only trivial phage multiplication (Fig. 6). Opacity curves (Fig. 7) of cultures of Q1 (A1*b*) exposed to A2*b* confirmed the poor plating efficiency. At the

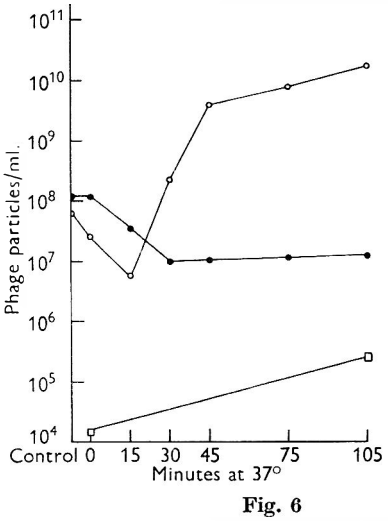

Fig. 6

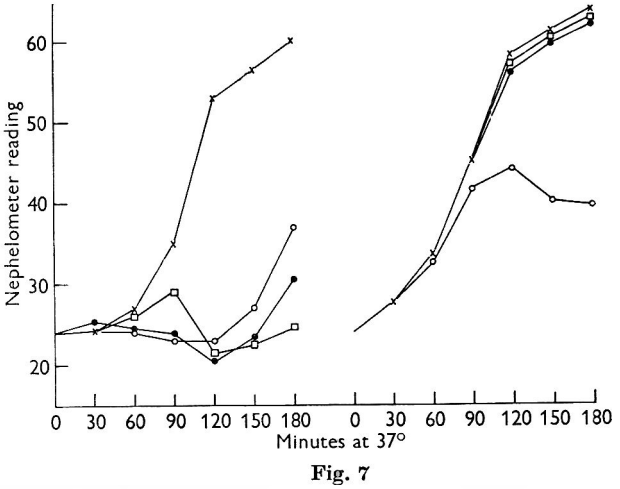

Fig. 7

Fig. 6. Adsorption of A2*b* on Q1 and Q1 (A1*b*); ○, on Q1, normal; ●, on Q1 (A1*b*), delayed, and only a minor degree of phage production; □, A1*b* in control culture of Q1 (A1*b*).

Fig. 7. Opacity curves of Q1 and Q1 (A1*b*) exposed to A2*b*. A2*b* was actively lytic against Q1. Against Q1 (A1*b*) the lytic action was seen only in the 10:1 ratio after 120 min. ×, control; ○, 10:1 ratio; ●, 1:1 ratio; □, 0·1:1 ratio.

lower ratios (0·1:1 and 1:1) the curves corresponded closely to the control and showed no clearing. At the 10:1 ratio clearing occurred between 120 and 150 min., indicating that at this phage concentration some vegetative development occurred. Attempts to demonstrate conversion by method 3 were unsuccessful; even at a 10:1 ratio all the colonies recovered were unchanged Q1 (A1*b*). Using method 1, 57 colonies of normal appearance were selected and tested. Forty-three were Q1 (A2*b*), 13 were Q1 (A1*b*) and one was 'cured'. No attempt was made to estimate the number of bursts, though the opacity curves show that lysis occurred. This is one of the cases in which, despite the negative results given by method 3, the conversions by method 1 in repeated experiments were unexpectedly high.

To summarize, A1*b* evicted and replaced prophage A2*b* in Q1 (A2*b*), but only to about 1/3 the extent to which it affected Q1 (A1*a*). A2*b* had a similar but weaker action against Q1 (A1*b*). In both cases 'cures' occurred.

*A*1b ⇌ *A*1c. Neither of these phages had any visible lytic action on the opposite lysogenic organism. The opacity curves showed some retardation in the higher

phage concentrations, but no clearing. In both cases adsorption was 90 % or over at a 1:1 ratio (Figs. 8, 9). Despite the absence of visible lysis, in both cases the rise in the free-phage titre, after a lag period of 45 min. or longer, showed that bursts had occurred, while the increase of free phage from the prophage of the lysogenic organism indicated that there had been some induction. Attempts to demonstrate prophage substitution by method 1 revealed a conversion rate of 1/20 when Q1 (A1*c*)

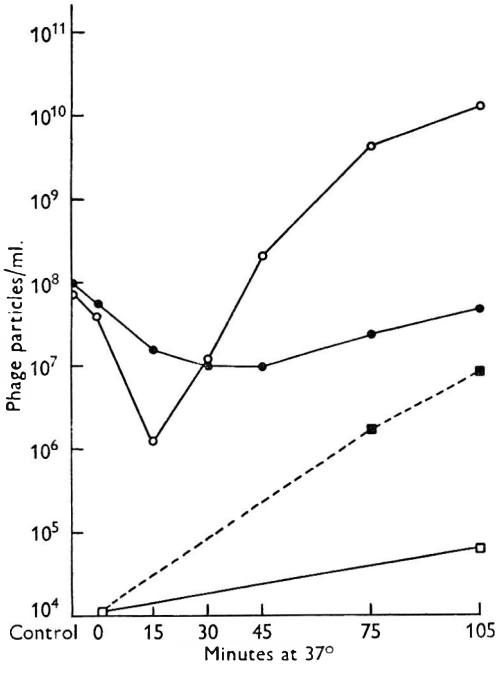

Fig. 8

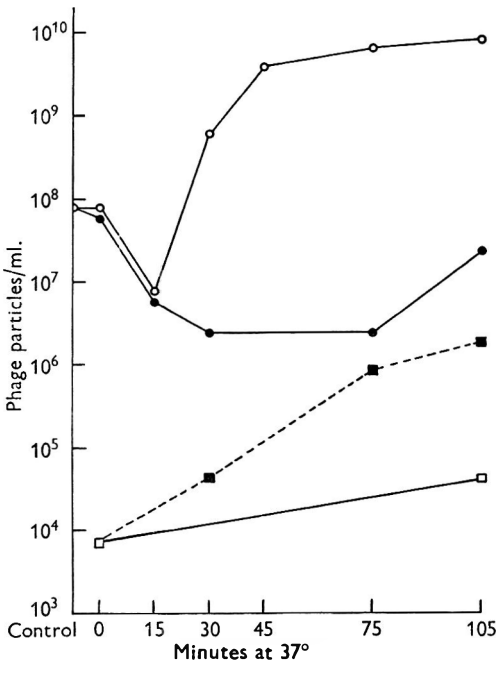

Fig. 9

Fig. 8. Adsorption of A1*b* on Q1 and Q1 (A1*c*). Adsorption on Q1 (A1*c*) was more limited than in the control Q1. Free phage production was registered after 45 min. incubation. There was some induction of Q1 (A1*c*). ○, A1*b*, adsorbed on Q1; ●, on Q1 (A1*c*). ■, Free phage from induction of Q1 (A1*c*). □, A1*c* in control culture of Q1 (A1*c*).

Fig. 9. Adsorption of A1*c* on Q1 and Q1 (A1*b*). Adsorption on Q1 and subsequent phage production was normal. Adsorption on Q1 (A1*b*) was normal, but phage production was delayed. Q1 (A1*b*) was induced to a limited extent. ○, A1*c*, Adsorbed on Q1; ●, A1*c* adsorbed on Q1 (A1*b*); ■, A1*b* from induction of Q1 (A1*b*); □, A1*b* in control culture of Q1 (A1*b*).

was superinfected with A1*b*, and 16/20 in the reverse reaction, A1*c* against Q1 (A1*b*). However, using method 3, A1*c* at a 10:1 ratio failed to effect prophage substitution in Q1 (A1*b*).

Double lysogenization

*A*2e ⇌ *A*1a. A2*e*, which has much the same spectrum of lytic action as A1*b*, gave double lysogenization with Q1 (A1*a*). The plating efficiency was not significantly lower on Q1 (A1*a*) than on Q1, the plaque counts of the batch of phage used in these experiments being $4{\cdot}4 \times 10^9$/ml. and $5{\cdot}0 \times 10^9$/ml. respectively. Adsorption

of A2*e* on Q1 (A1*a*) was as active and rapid as on Q1 (Fig. 10), but in both cases free phage production was delayed. The opacity curves of infected cultures revealed lysis in the lower concentrations, which was more marked in the Q1 cultures than in Q1 (A1*a*), particularly in the 0·1:1 ratio.

As the Levine technique cannot be used in double lysogenizations, the degree of double lysogenization and lysis resulting from the superinfection of Q1 (A1*a*) with

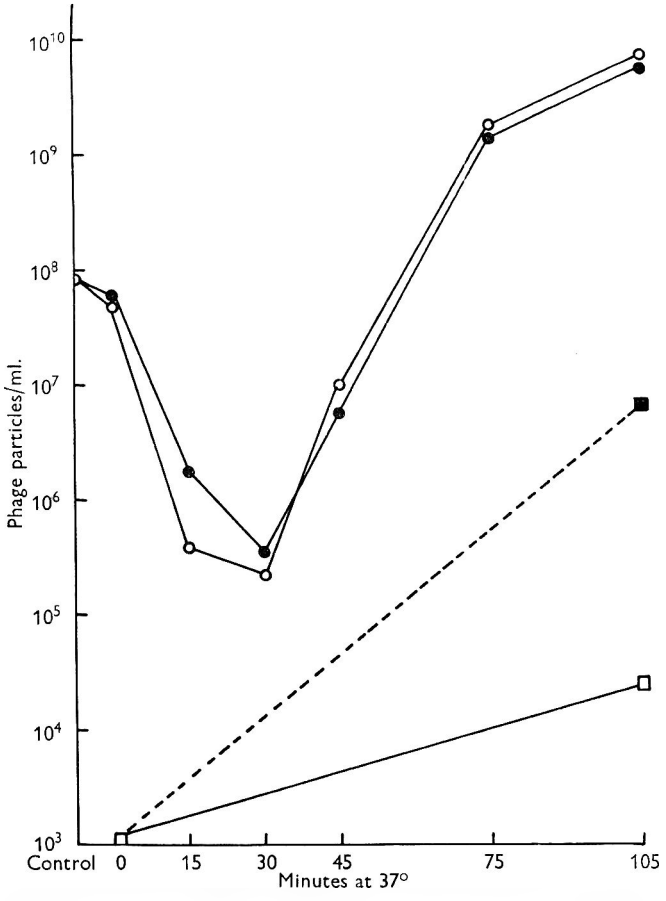

Fig. 10. Adsorption of A2*e* on Q1 and Q1 (A1*a*). There was no significant difference between the two curves. Adsorption was active, free phage production slightly delayed. There was some induction of Q1 (A1*a*). ○, Adsorption of A2*e* on Q1; ●, on Q1 (A1*a*); ■, A1*a* from induced Q1 (A1*a*). □, A1*a* in control culture of Q1 (A1*a*).

A2*e* was determined by method 3. The results are recorded in Table 4, where they are compared with the corresponding figures given by Q1 infected with the same batch of A2*e*. In the lower ratios, there was no significant difference. In the 10:1 ratio there were fewer bursts, and more lysogenizations on Q1 than there were bursts and double lysogenizations on Q1 (A1*a*). Boyd & Bidwell (1961) showed that A2*e* produces a lower percentage of lysogenizations in Q1 than does A1*b*. Taking this into consideration, A1*b* and A2*e* are alike in the way they affect

Q1 (A1*a*), except that A1*b* gives prophage substitution and A2*e* double lysogenization. Prophage A1*a* confers on its host bacterium little if any immunity against either of these superinfecting phages. In the reverse reaction, although there was no visible lytic action, A1*a* was well adsorbed to Q1 (A2*e*), about 95% being removed in 30 min., from a mixture having a 1:1 ratio. There was no evidence

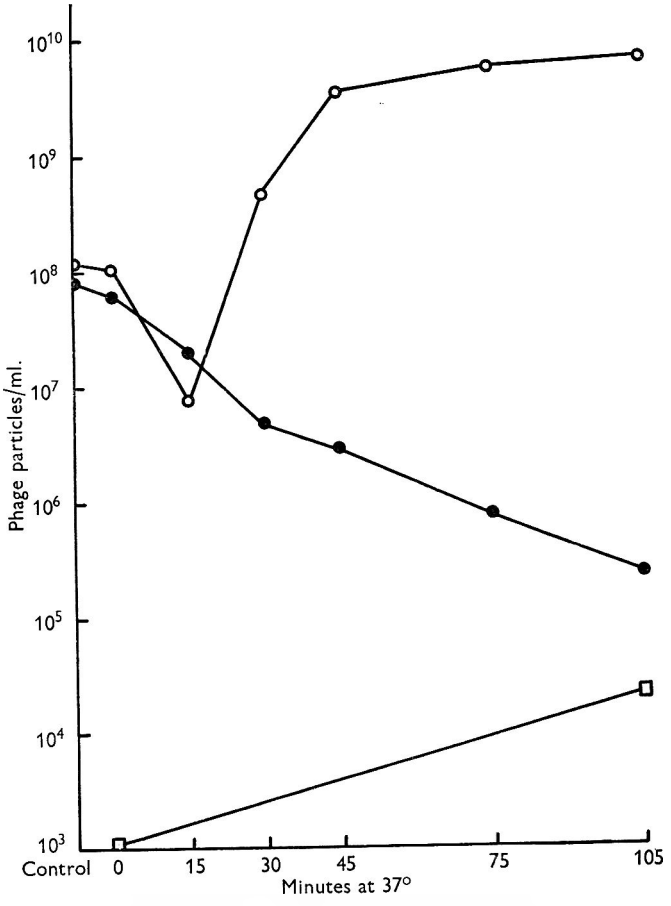

Fig. 11. Adsorption of A1*a* on Q1 and Q1 (A2*e*). Adsorption was delayed. There was no phage production. ○, On Q1; ●, on Q1 (A2*e*); □, A2*e* in a culture of Q1 (A2*e*).

Table 4. *Comparison of* Q1 *infected and* Q1 (*A* 1 a) *superinfected with A* 2 e

As in Table 3, except that the technique of method 3 was followed. In the 0·1:1 ratio of superinfection no conversions of Q1(A1*a*) were found in 128 colonies examined. A2*e* was the only phage present in the bursts.

Phage:bacteria ratio ...	0·1:1		1:1		10:1	
Phage A2*e* infecting or superinfecting	Q1	Q1 (A1*a*)	Q1	Q1 (A1*a*)	Q1	Q1 (A1*a*)
Lysogenics	1·1	0/128	11	11·3	57·7	36
Bursts	10	8·4	36	30	4·4	8

of vegetative development in the later stages of this experiment (Fig. 11). Using method 1, 40 colonies were examined; only 3 showed double lysogenization. This reaction, though present, was therefore weak. In this and other similar experiments with these phages no cures were found.

A 1b $\rightleftharpoons$ *A*2c. A1*b* and A2*c* each had a weak lytic action on the opposite lysogenic organism. The plating efficiency of A1*b* on Q1 (A2*c*) was considerably less than on Q1. Thus a batch having a plaque count of $1{\cdot}6 \times 10^{10}$/ml. on Q1 gave a

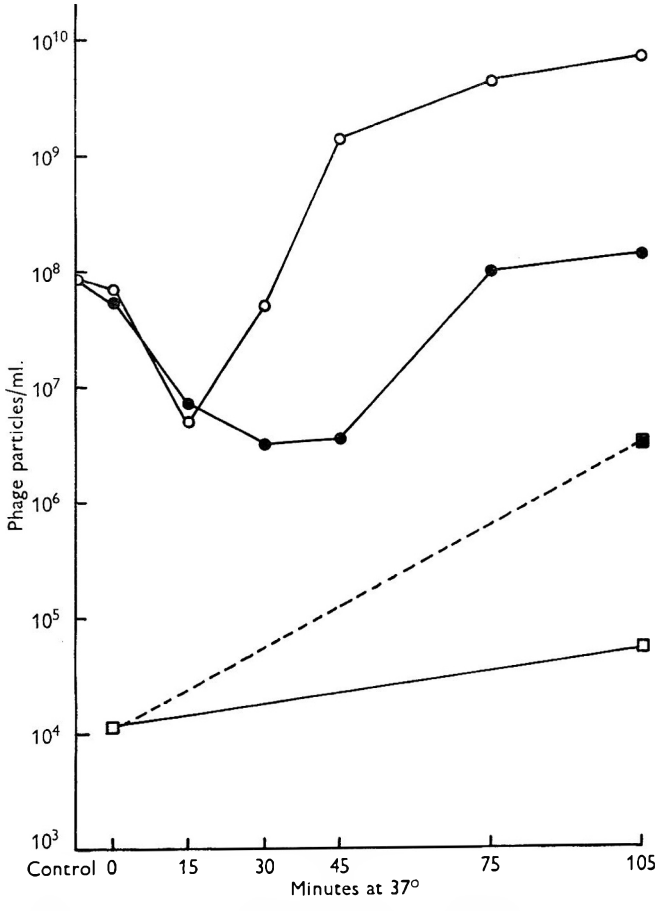

Fig. 12. Adsorption of A1*b* on Q1 and Q1 (A2*c*). Adsorption on Q1 (A2*c*) was as active as on Q1, but phage production was delayed for 45 min. Q1 (A2*c*) showed some induction. ○, Adsorption of A1*b* on Q1; ●, adsorption of A1*b* on Q1 (A2*c*); ■, A2*c* from induction of Q1 (A2*c*); □, A2*c* in control culture of Q1 (A2*c*).

count of $7{\cdot}7 \times 10^{7}$/ml. on Q1 (A2*c*). The plaques on Q1 (A2*c*) were shallow, and varied considerably in size, some being so small that they could only be seen by transmitted light, using a plate microscope. Adsorption was similar to that on Q1, but phage production did not occur for 45 min. There was some induction of Q1 (A2*c*) (Fig. 12). Opacity curves of Q1 (A2*c*) superinfected with A1*b* showed some retardation of growth but no clearing in the 10:1 and 1:1 ratios; in the 1:10

ratio slight clearing occurred after 150 min. incubation. In a superinfection experiment, carried out by method 3 at a 1:1 ratio, 2·5 % of the Q1 (A2*c*) bacteria were doubly lysogenized, i.e. converted to Q1 (A1*b*, A2*c*). Bursts were not estimated and no cures were found in 130 colonies which were examined. In view of the poor plating efficiency the relatively high conversion rate is notable. Method 3 gave better results than method 1 (compare Q1 (A1*b*) superinfected by A2*b*).

The plating efficiency of A2*c* on Q1 (A1*b*) was considerably higher than in the reverse reaction. The plaques were nevertheless much smaller (half size or less) and shallower than corresponding plaques on Q1. The respective plaque counts of the batch tested were $1{\cdot}44 \times 10^{10}$/ml. on Q1, and $1{\cdot}14 \times 10^{9}$/ml. on Q1 (A1*b*). Adsorption was good, being little different from adsorption on Q1, but, as would

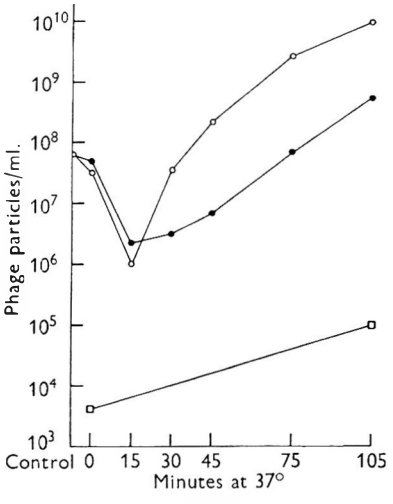

Fig. 13

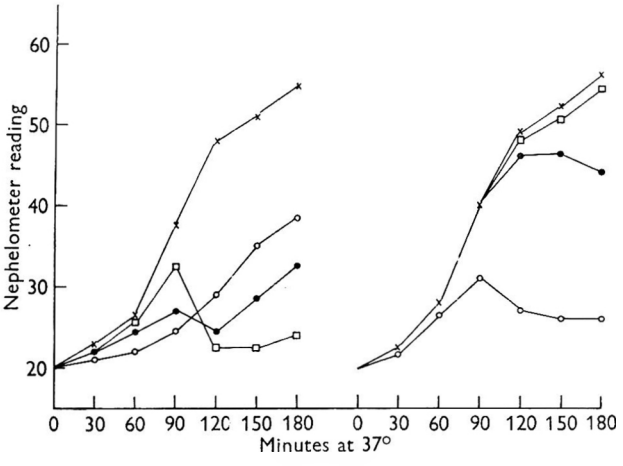

Fig. 14

Fig. 13. Adsorption of A2*c* to Q1 and Q1 (A1*b*). Adsorption on Q1 (A1*b*) was similar to the control, but free phage production was delayed. ○, Adsorption of A2*c* on Q1; ●, adsorption of A2*c* on Q1 (A1*b*); □, A1*b* in control culture of Q1 (A1*b*). There was no significant induction of A1*b*.

Fig. 14. Opacity curves of cultures of Q1 and Q1 (A1*b*) exposed to A2*c*. Against Q1 (A1*b*), A2*c* had no action at 0·1:1 ratio, showed delayed clearing at 1:1, and at 10:1 gave clearing similar to 0·1:1 in the control against Q1. ×, Control; ○, 10:1 ratio; ●, 1:1 ratio; □, 0·1:1 ratio.

be expected, subsequent phage production was less active (Fig. 13). The opacity curves (Fig. 14) showed clearing in the 10:1 and 1:1 ratios. Using method 3 at a 1:1 ratio, no prophage change was detected: at 10:1, 13·6 % of the bacteria were doubly lysogenized. No cures were found.

*A*1b ⇌ *A*2e. The two phages A1*b* and A2*e*, both active against several other lysogenic strains, were cross-immune in terms of detectable lytic action, but when examined by method 1, each phage was found to produce double lysogenization in the opposite lysogenic strain, though A2*e* as a superinfecting phage was more

active than A1*b*. In a mixed culture of the doubly lysogenic strains and Q1, the predominant free phage, irrespective of the origin of the strain (whether A1*b* on Q1 (A2*e*) or vice versa) was A1*b*. In early experiments the presence of A2*e* was missed because the propagating culture was not incubated for a sufficiently long time.

Interaction of phages of different antigenic structure

Ten of the twelve phages under consideration—those of the A1 and A2 series—have a similar antigenic structure, while A3 and A4 have distinctive antigens, and also differ from the A1 and A2 phages in that they markedly reduce the capacity of the bacteria they lysogenize to adsorb homologous and heterologous phages

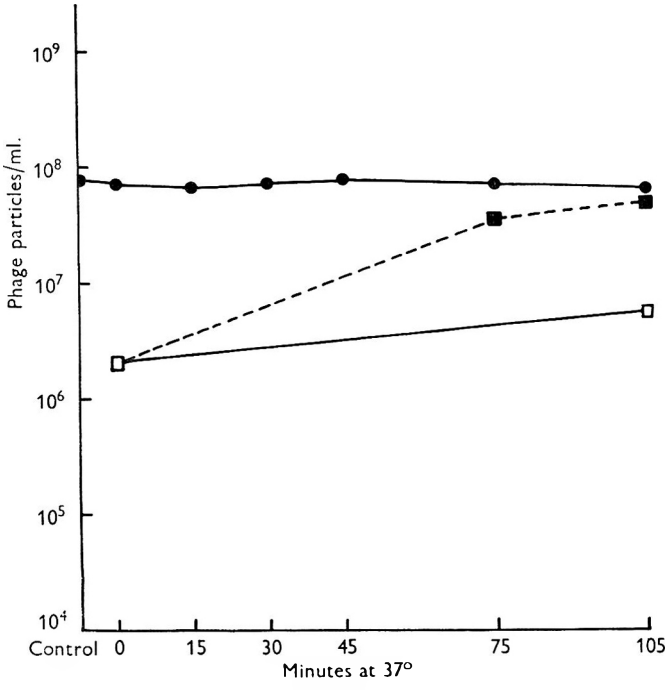

Fig. 15. Adsorption of A3 on Q1 (A1*b*) and of A1*b* on Q1 (A3). A3 was freely adsorbed on Q1 (A1*b*) without any production of free phage. Adsorption of A1*b* on Q1 (A3) was too trivial to be detectable by this technique, yet after an interval of 45 min. free phage was produced, showing that adsorption and lysis had occurred. ●, A3 on Q1 (A1*b*); ○, A1*b* on Q1 (A3).

(Boyd, 1954). This was less obvious in the case of Q1 (A3) which despite its poor adsorptive properties showed, when superinfected with the other phages, a good spectrum of lysis with a more limited degree of prophage change. In Fig. 15 the adsorption of A1*b* on Q1 (A3) is shown in comparison with the reciprocal reaction. The latter followed the normal pattern, while in the former there was no detectable reduction of free phage particles, and the only evidence of adsorption was their increase after an interval of 45 min., presumably the outcome of productive development. Other members of the group reacted in the same way, as can be seen in respect of A2*b*, A2*d* and A2*e* in Fig. 16. The visible lysis produced by

method 1 was well marked, and in comparison with this, prophage change was weak. Table 5 gives the percentages of bacteria showing prophage change when superinfected in a 10:1 ratio, using method 3.

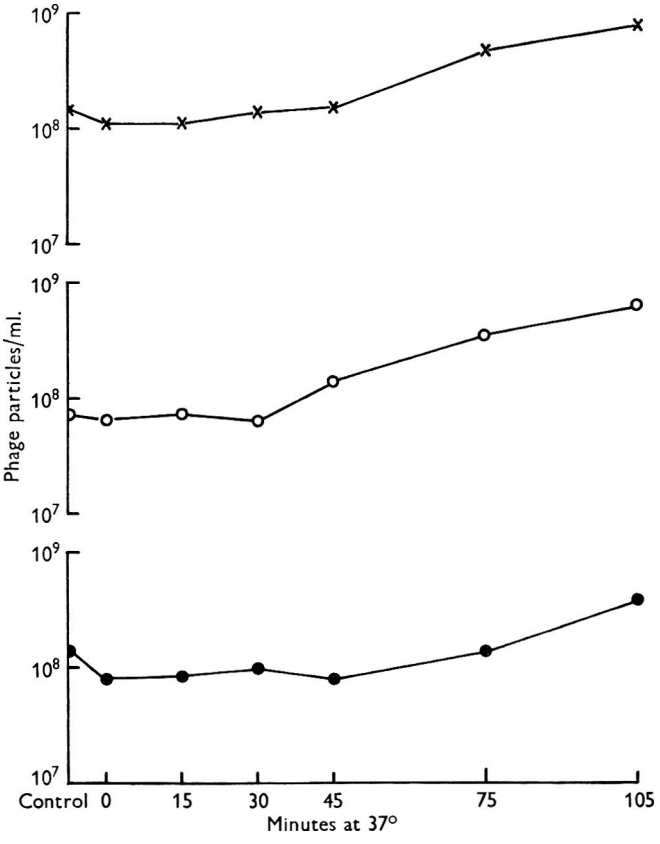

Fig. 16. Adsorption of A2*b*, A2*d*, and A2*e* on Q1 (A3). All three were feebly adsorbed, but gave delayed liberation of free phage. ×, A2*b*; ○, A2*d*; ●, A2*e*.

Table 5. *Prophage change in superinfected* Q1 (A3)

The technique of method 3 was followed.

	Superinfection of Q1 (A3) in a 10:1 ratio by phages										
	A1*a*	A1*b*	A1*c*	A1*d*	A2*a*	A2*b*	A2*f*	A4	A2*c*	A2*d*	A2*e*
Percentage of Q1 (A3) showing prophage change	0	3·6	0	2	2·4	0·7	0	0	3·4	1·25	6·6

The characters present in A3 are more strongly marked in A4. Selecting as an example its reciprocal action with A1*b*, A4 was freely adsorbed to Q1 (A1*b*) whereas the adsorption of A1*b* to Q1 (A4) was trivial (Fig. 17), and was followed by only a feeble rise in titre which is in general agreement with the weak lytic

action shown in Fig. 1. We have failed in repeated tests to provoke prophage substitution in Q1 (A4) by superinfection with A1*b*. Conversely A4, though producing no visible lysis in Q1 (A1*b*), was capable of evicting and replacing its prophage.

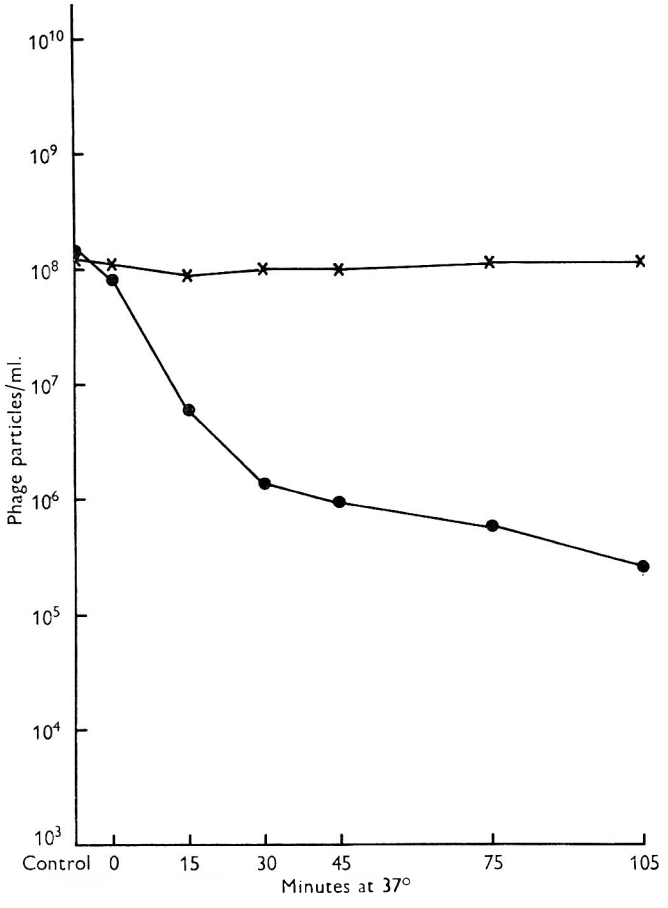

Fig. 17. Adsorption of A1*b* on Q1 (A4) and A4 on Q1 (A1*b*). A4 was freely adsorbed on Q1 (A1*b*), without production of free phage. A1*b* was weakly adsorbed on Q1 (A4), with slight production of free phage. ×, A1*b* on Q1 (A4); ●, A4 on Q1 (A1*b*).

Superinfections of Q1 (A4) with A1*b*, A2*c*, and A2*d* were the only instances encountered in this series in which lysis occurred unaccompanied by detectable prophage change. It was deemed of interest to determine whether the superinfecting phage was multiplying in Q1 (A4) or was inducing prophage A4, and burst experiments were carried out with A1*b*, A2*c*, A2*d* and also A2*e* (Table 6). Apparently these superinfecting phages may either multiply in Q1 (A4) without inducing the prophage, or conversely may induce the prophage without themselves multiplying. In a few instances both phages have been found in the same tube, but as some of the tubes must have contained more than one bacterium, it cannot be concluded that in such cases both phages multiplied together in one host organism. The plaque

characters of A4 are distinctive, and consequently the identification of the two types presented no difficulty. In only one of the bursts was there evidence of hybridization.

The only instance of prophage substitution in Q1 (A4) was in superinfection with A2*b*, where two colonies of Q1 (A2*b*) were found in sixty examined.

The action of A2*e* on Q1 (A4) was of especial interest. A2*e* had a spectrum of lytic activity closely resembling that of A1*b*. It was aggressive in producing lysis and lysogenesis, and in its prophage phase afforded a good degree of protection against lysis by the other members of the series, but less against prophage change, which in the appropriate grouping occurred either as substitution or as double lysogenization. However, A2*e* differed from A1*b*, and indeed from all the other phages of the group, in that it had a well-marked lytic action on Q1 (A4), mainly due to induction (Table 6 and Fig. 18), and also produced a considerable degree of double lysogenization. This was not associated with better adsorption (Fig. 18, compare with Fig. 17).

Table 6. *Superinfection of Q1 (A4): analysis of bursts*

Superinfection	Phage: bacteria ratio	Estimated bacteria per tube	Nos. of plaques in bursts		
A1*b* on Q1 (A4)			A1*b*	A4	
	1:1	1 bacterium in 4 tubes	78	—	
			120	—	
			6	244	
	1:1	1 bacterium in 1 tube	120	—	
			4	176	
			100	—	
			134	—	
			240	—	
			12	188	
			58	50	
			160	—	
			—	168	
			—	64	
			—	164	
			4	170	
	4:1	1 bacterium in 4 tubes	—	62	
			2	84	
			—	56	
A2*c* on Q1 (A4)			A2*c*	A4	
	1:1	1 bacterium in 1 tube	976	—	
			204	—	
			234	—	
A2*d* on Q1 (A4)			A2*d*	A4	
	1:1	1 bacterium in 1 tube	90	—	
	10:1	1 bacterium in 4 tubes	4	44	
A2*e* on Q1 (A4)			A2*e*	A4	? hybrids
	1:1	1 bacterium in 2 tubes	—	48	
			—	40	
			4	122	
			8	654	
			108	12	38

Cures

In carrying out prophage substitution experiments by method 1, we isolated non-lysogenic, i.e. 'cured', bacteria on a number of occasions, particularly from Q1 (A1*b*). The reactions in which these occurred are not fully recorded in this paper, as the phenomenon has not been investigated with sufficient thoroughness. On only one occasion (Q1 (A4) superinfected by A2*e*) did we encounter cure in association with double lysogenization.

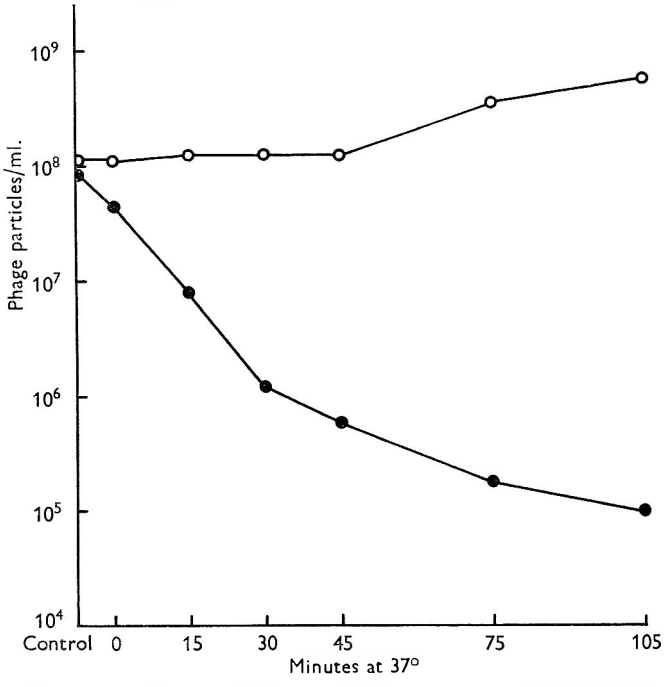

Fig. 18. Adsorption of A2*e* on Q1 (A4). The curve gave certain evidence neither of adsorption nor of multiplication of A2*e*. There was, however, some induction of Q1 (A4). ●, A2*e* on Q1 (A4); ■, free phage A4 from induction of Q1 (A4); □, A4 in control culture of A4.

DISCUSSION

The problems of prophage substitution and double lysogenization were first studied by Bertani (1953, 1954) who worked with strains of phage isolated from *Escherichia coli* Li. In all but his first experiments the 'wild' form of one of these strains, P2, was used together with mutants obtained from it by irradiation*: the mutants could be distinguished from the wild form by their plaque characters. A strain of *Shigella dysenteriae* (Sh) served as indicator. When Sh (*P*2) was superinfected with one of the mutants, all the cells survived superinfection, though after a few generations a percentage of superinfected cells 'burst' liberating phages of both types. In certain cases both prophage substitution and double lysogenization were found, while, after several hours incubation, cells were isolated in which

* We have recently been informed by Dr Bertini that in his earlier work the mutants he used occurred spontaneously and not as the result of irradiation.

segregation had not occurred, and daughter cells containing one or other of the prophages continued to be produced. A few recombinants were detected. Following up this work, Bertani & Six (1958), using these same phages and another, P2 Hy dis (Cohen, 1959), together with a large number of artificially produced mutant strains of *Escherichia coli* C, *E. coli* K12, and *Shigella dysenteriae*, found that there were several chromosomal sites to which these phages could become attached, one the preferred location, the others second-choice locations. Six (1960, 1961) extended these observations, and found that genetic incorporation occurred most readily in the preferred location, either by prophage addition or substitution, and that the frequency of these changes increased linearly with multiplicity. It is to be noted that these experiments were carried out with mutants of one parent phage; all were therefore very closely related.

Groman (1955) and Groman & Eaton (1955) experimented with phages recovered from *Klebsiella diphtheriae*. Their object was to demonstrate the relationship of toxigenicity to lysogenization with one of these phages, but in doing so they produced evidence of prophage substitution, of double lysogenization, of recombination and of 'cure'. Their findings differ from those of Bertani and Six in that all the phages used were 'wild'. Gorrill (1957) reported prophage substitution and double lysogenization with staphylococcal phages. Zinder (1958) studied lysogenization and superinfection immunity in Salmonella but did not specifically investigate prophage substitution and double lysogenization. He found that when a prophage did not confer immunity to superinfection with a related phage, the same general effects occurred as those following infection of a non-lysogenic strain. He suggested that the first step is comparable to U.V. induction.

The observations recorded in this paper, on which a brief preliminary note was published (Boyd, 1956), differ from those of other workers in that they concern a relatively large group of closely related phages, all of which, apart from adaptation to a common host bacterium, are in the 'wild' state (Boyd & Bidwell, 1957). To what extent they are descended from a common source can only be a matter for speculation. They were found in lysogenic strains of *Salmonella typhimurium* isolated in different laboratories throughout the British Isles from faeces, contaminated food, etc. A few came from overseas, and there is little doubt that these and other related phages have a world-wide distribution. The prophage change which has been observed in laboratory experiments may well occur in natural surroundings if lysogenic bacteria carrying different types of phage are brought into close proximity, and in this way new types may arise from the emergence of recombinants.

Although the end-result of superinfection depends on the properties of both superinfecting phage and lysogenic bacterium, a better idea of the part played by each of the participants in the reaction is to be obtained by considering them separately.

The varying properties of the superinfecting phage

Aggressive and non-aggressive phages. There was considerable difference in the aggressiveness of the different phages (Fig. 1, Table 1). Some (A1*b*, A1*c*, A2*e*), appeared to dominate many of the lysogenic strains, and brought about well-marked lysis and prophage change; others (A1*a*, A2*f*, A3) were, in terms of lysis, apparently inactive, and produced only feeble prophage change; the remaining six occupied intermediate positions. This pattern of action was not consistent, however, and sometimes the so-called weak phage gave a strong reaction where a strong phage reacted weakly. Thus A2*b* and A2*d* produced well-marked lysis of Q1 (A2*c*), against which the aggressive A1*c* and A2*e* were inactive, whilst A1*b* gave only faint lysis. A2*b* lysed Q1 (A1*c*), A1*b* did not. A2*c* lysed Q1 (A1*b*), A1*c* did not. Other minor examples can be seen in Fig. 1.

Relationship of lysogenizing and lytic action. In the early stages of this work it was assumed that prophage change would be found in lysogenic bacteria superinfected with heterologous phage only where visible evidence of coincident vegetative development was demonstrated by the formation of plaques. Doubt was cast on this assumption by the discovery that in some cases (e.g. Q1 (A1*b*) superinfected with A2*c*) the degree of prophage change bore no relation to the plaquing efficiency of the particular phage on the lysogenic organism. In certain pairs (e.g. A1*a* and A1*b*) in which the cross-immunity test, based on plaquing, gave a one-way reaction, it could be shown that the prophage change was two-way, although much less active in the lysogenic strain which appeared resistant to lysis by the opposite phage. Observations were then extended to pairs which, in terms of plaquing, seemed to show complete cross-immunity. It was found that a limited degree of prophage substitution or double lysogenization sometimes occurred. If a sufficiently exhaustive examination were made, some prophage change could probably be detected in many of the interactions now recorded as negative in Fig. 1. Nor can the absence of visible plaque formation always be taken as proof that there has been no vegetative development either of the superinfecting phage or of induced prophage. Trivial degrees of prophage change can be detected by method 1, but we have no corresponding simple technique to detect minor degrees of vegetative development, though the late upward trend of the phage titre in some adsorption tests (Figs. 8, 9) indicated that lysis had occurred.

Superinfecting phages which gave well-marked lysis were usually active in producing prophage change while non-aggressive ones were not. Thus the aggressive phages A1*b*, A1*c*, A2*e*, and to a lesser extent A2*b* and A2*d*, which had a wide range of lytic activity, effected a considerable degree of prophage change. To this generalization there were exceptions, e.g., in superinfection of Q1 (A1*c*) with A2*b*, there was a moderate but constant degree of lysis together with an almost complete absence of prophage change. Conversely, the non-aggressive phages A1*a*, A2*f* and A3 which with one minor exception showed no lytic activity, effected very little prophage change.

The significance of the antigenic structure of superinfecting phage

The antigenic structure of the phage had no obvious bearing on its superinfecting capacity. A3 was found to have a spectrum of action very similar to that of A1*a* and A2*f*, while A4 had weak activity not unlike A1*d* and A2*a*.

Evidence of different sites of attachment to the chromosome

The most striking feature of these findings was the sharply defined pattern of prophage substitution and double lysogenization which split the series into two groups (Fig. 1). The larger, group 1, comprised A1*a*, A1*b*, A1*c*, A1*d*, A2*a*, A2*b*, A2*f*, A3 and A4. The smaller, group 2, comprised A2*c*, A2*d*, and A2*e*. The type of prophage exchange between any two members of group 1 or any two members of group 2, with exceptions which do not affect the significance of the observation, was prophage substitution. When lysogenic strains of group 1 were superinfected with phage of group 2, or vice versa, double lysogenization resulted. This amply confirmed observation suggests that there are two sites on the bacterial chromosome to which the infective element of these phages can become attached as prophage, and that each group is restricted to one of these sites. Thus superinfecting phages of either group were able to establish themselves in heterologous lysogenic strains of the same group only by dislodging the resident prophage. On the other hand, superinfecting phages of group 1, by attaching themselves to the vacant site in the lysogenic bacteria of group 2, produced double lysogenization, while group 2 phages reacted similarly with group 1 bacteria. There was one apparent deviation from this rule which requires some explanation. As superinfecting phage, A2*f* behaved as a member of group 1: as prophage in Q1 (A2*f*) it was evicted and replaced by superinfecting phages of both groups except on one occasion, when superinfection with A2*d* gave double lysogenization. However, the double lysogenizations which superinfections with A2*f* produced in members of group 2 showed clearly that A2*f* must occupy a different site from the group 2 prophages. A possible explanation of the anomalous findings is that, while A2*f* as an invading phage could establish itself on the vacant site, as resident prophage its attachment was insecure, so that it was displaced by the invading phages as they became attached to the alternative site. Another possibility is that prophage A2*f* occupied a slightly different site from the other group 1 prophages, in closer proximity to the group 2 site, and because of this was more liable to be dislodged.

The varying properties of the lysogenic bacterium (bacterium–prophage complex)

Variations in the degree of immunity. In general terms, the aggressive phages (A1*b*, A1*c*, A2*e*), when present in the host bacterium as prophage, offered a considerable degree of resistance to most superinfecting phages, and to that extent conferred immunity to both lysis and lysogenesis (see Fig. 1, Table 1). On the other hand, the non-aggressive phages (A1*a*, A2*f* and A3) had little or no protective action other than against one another, while strains A1*d*, A2*a*, A2*b* of group 1 were intermediate. A4 is in a category by itself, and will be discussed separately. The two remaining intermediate strains, A2*c* and A2*d*, which belong to group 2, produced, as prophages, a wider range of immunity than did the corresponding strains in group 1.

The results given by superinfection with A1*b* of all the lysogenic strains (Fig. 1, Table 2) provide a detailed example of the varying degrees of immunity to one particular phage which the different prophages can confer. Resistance to prophage change when exposed to a 1:1 ratio varied from 100% in Q1 (A1*c*), Q1 (A4), Q1 (A2*d*) and Q1 (A2*e*) to nil in Q1 (A1*a*), which exhibited no resistance of any kind and gave 'multiplicity infection' results corresponding closely to those given by Q1 (Table 3). This strain was equally susceptible to superinfection with A2*e* (Table 4).

The most striking feature overall was the irregularity of the reactions. Each lysogenic strain had its own idiosyncrasies.

Variations in the type of resistance offered by different lysogenic bacteria

Usually, but not invariably, resistance to lysis and resistance to prophage change were roughly parallel. However, resistance to lysis was sometimes greater than resistance to prophage change, when both were considerably reduced (Fig. 1, Table 1). In this respect the reactions of Q1 (A1*b*) and Q1 (A1*c*) are of interest. Both were strongly resistant to lysis though each showed two minor breakdowns. But whereas Q1 (A1*c*) resisted prophage change completely in 7 and almost completely in a further 3 of the 11 superinfections, Q1 (A1*b*) was relatively susceptible, and its prophage was evicted and replaced even by the non-aggressive phages A1*a*, A2*f*, and A3. The vulnerability of Q1 (A1*b*) to these three phages is the more remarkable when it is recalled that they were cross-resistant to each other. It is of interest to recall that Q1 (A1*b*) and Q1 (A1*c*) differ in their response to u.v. irradiation (Boyd & Bidwell, 1959). The reaction of Q1 (A1*b*) when superinfected with A1*a* is a good example of immunity to lysis but not to prophage change (Table 1, Fig. 4, and text). A2*e*, the third of the aggressive phages, behaved in much the same way as A1*b*, giving a good degree of protection against lysis but less against prophage change.

In contrast, resistance to prophage change can exceed resistance to lysis. This is seen in the reactions of Q1 (A3) and Q1 (A4), which, it will be noted, were completely cross-resistant to superinfection with their reciprocal phages. Apart from immunity to the non-aggressive phages A2*a* and A2*f*, Q1 (A3) showed well-marked lysis with only a limited degree of prophage change when superinfected with the remaining 8 phages (Fig. 1, Table 5). In Q1 (A4) resistance to both lysis and prophage change was greater than in Q1 (A3) and in 3 instances lysis occurred without any accompanying prophage change. Q1 (A3) and Q1 (A4) were alike in having very limited powers of adsorption (Figs. 15, 16, 17, 18), presumably because the prophage interferes in some way with the genetic mechanism which controls the bacterial surface. As a sequel to poor adsorption, the multiplicity effect (Boyd, 1951) must be partly nullified, with a consequent bias towards vegetative development. It is unlikely, however, that this plays a significant part in producing the unusual results. It does not account for the marked difference between the reactions of Q1 (A3) and those of Q1 (A4) in both of which adsorption was equally restricted, nor for the reactions of Q1 (A4) when superinfected with A2*e*. A2*e* was no better adsorbed by Q1 (A4) than the other phages of the series, yet it produced well-marked lysis and a good degree of double lysogenization. This suggests the existence of a barrier or repressor, distinct from defective adsorption, which A2*e*, but not the others, was able largely to evade. The presence of such a repressor, more active

in Q1 (A4) than in Q1 (A3) and directed more against prophage change than against vegetative development, is a plausible if hypothetical explanation of the phenomenon.

Q1 (A1*c*) was completely resistant to A2*c*. This, apart from the reactions of Q1 (A2*f*), which have already been discussed, was the only instance of resistance to prophage change where double lysogenization would be expected.

In tests using method 1, lysogenic bacteria were exposed to high concentrations of superinfecting phage, and consequently each bacterium was invaded by several, perhaps many phage particles. (This statement does not apply to Q1 (A3) and Q1 (A4) which have restricted powers of adsorption.) Despite this heavy infection, many of the bacteria did not react, and sometimes only a small percentage underwent either lysis or prophage change. What is the cause of this breakdown of immunity in occasional members of a population which is otherwise resistant—a non-resistant bacterium, a more than ordinarily aggressive phage particle, or the cumulative effect of multiple infection? The available evidence suggests that it is the last of these possibilities. On several occasions we have found prophage change when using method 1 where repeated tests by method 3, in which many bacteria were examined, gave negative results. The significant difference between the methods is that in the former there is a heavy multiplicity of infection while in the latter superinfected bacteria adsorb a relatively small number of particles. The property which confers resistance is probably limited, and can be neutralized by weight of numbers. In these experiments the distribution of particles per bacterium varied around a mean, and the most likely explanation of the occasional positive reactions is that the bacteria involved were so heavily infected that the resistance was saturated and one or more particles left to develop unimpeded. This theory leaves unexplained the few cases in which method 3 gave more favourable results than would be expected from the results given by method 1, as in superinfection of Q1 (A2*c*) by A1*b*. This unexpected finding may be related to lysis from without but requires further investigation.

The mechanism of immunity

The main interest of these observations lies in the information they provide concerning the mechanism of the immunity of lysogenic bacteria to homologous or heterologous phages. There is no convincing evidence that immunity is related to defective adsorption. The non-aggressive phages of group 1, which have minimal lysogenizing and lytic action, were as freely adsorbed as the aggressive phages which produced marked lysis and prophage substitution. Conversely, adsorption to Q1 (A3) and Q1 (A4) was equally restricted whether the superinfecting phage was active or not. Nor can any major role be attributed to interference resulting from the presence of the prophage on the bacterial chromosome in such a position that it prevented the invading heterologous phage from gaining access to this particular and essential location. It is true that, while there were numerous negative findings in the blocks where prophage substitution would be expected to occur, there were very few in the series showing double lysogenization (Fig. 1). This might indicate that there was less opposition to double lysogenization than to prophage substitution—i.e. that it was easier for a superinfecting phage to occupy a vacant chromosomal site than to evict and replace a resident prophage. However,

the low incidence of lysis and prophage change in most cases where double lysogenization occurred, particularly in the superinfection of group 2 lysogenic bacteria with group 1 phages, showed clearly that the mere existence of a vacant site did not leave the door open for the invader, and that other and more potent forces were concerned in producing immunity.

Jacob & Campbell (1959), studying phenomena encountered in the zygotic induction of *Escherichia coli* K 12 (λ) by certain mutants of λ, suggested that a certain region, C 1, of the chromosome of this phage regulated the synthesis of a cytoplasmic element which might be either an enzyme geared to destroy a component formed by non-lysogenic bacteria which is specifically necessary for the vegetative development of phage λ, or a repressor which specifically inhibits one or more early reactions necessary for the multiplication of phages possessing the region C 1 of λ. The fate of a bacterium infected by λ would depend on the respective speed of the two processes, the synthesis of the repressor allowing of lysogenization on the one hand, and reactions which determine the triggering off of the vegetative phase on the other hand. They instance the action of chloramphenicol, which blocks protein synthesis and increases the probability of lysogenization, and moot the possibility of the repressor synthesized by the phage genome having a similar action. Lwoff (1961), discussing the immunity of a lysogenic bacterium to a mutant homologous phage, attributes this immunity to a specific repressor in the cytoplasm of the lysogenic bacterium which blocks specifically the initiation of the vegetative phase; here the question of prophage change does not arise, or at least is not obvious. Neither of these theories covers the interaction of lysogenic bacterium and superinfecting heterologous phage. If the curtailment of protein synthesis does in fact direct the superinfecting phage towards lysogenization and away from vegetative development, this cannot be the operative factor where both lysis and lysogenesis are suppressed. Our findings show that in many cases the repressor, whatever it may be, has this double action, and therefore appears to operate against some fundamental property common to all invading phages, whether destined to lysogenize or lyse. In certain cases, however, the repressor was more active against lysis, in others against lysogenesis. This, together with the irregularity and unpredictability of the reaction (well demonstrated by the reaction of Q 1 (A 4) superinfected with A 2*e*, Q 1 (A 2*c*) superinfected with A 2*b* and A 2*d*, and present though less obvious in some other instances) suggests that each lysogenic strain may possess a repressor or repressors with specific characters rather than that all produce, with quantitative variations, a common type of repressor. It might be argued that as the end-result depends on the interaction of repressor and invading phage, the anomalous results could stem solely from an unusual degree of resistance or virulence on the part of the phage. Such a theory, which implies the presence in each lysogenic strain of varying concentrations of a common repressor as well as varying degrees of aggressiveness in the different phages, fails to explain why, for example, A 1*c* is active against Q 1 (A 2*a*) and is almost completely suppressed by Q 1 (A 2*c*), whereas A 2*b* is well suppressed by Q 1 (A 2*a*) and active against Q 1 (A 2*c*). While, therefore, different phages clearly have different degrees of aggressiveness, it is equally certain that the different lysogenic strains contain repressors of varying degrees of specificity.

The outstanding feature of these findings is the complexity of the picture which they reveal. As the host bacterium is common to all the lysogenic strains, the

differing reactions must depend on variations in the phage component of the phage-bacterium complex, and this despite the fact that certain basic characters of these phages show them to be closely related members of a well-defined group. Prolonged study has failed to reveal a formula which would enable any given set of reactions to be predicted with certainty: they appear to be in a category inviolate to statistical calculations and mathematical equations.

The possible significance of the bacteriophage model

In man and other animals the immunity conferred either by an attack of a virus disease or by the administration of an attenuated but living virus vaccine, and also certain associated phenomena of viral interference, raise problems of great interest. Animal viruses undoubtedly differ in many fundamental respects from bacterial viruses, just as metazoan cells differ from bacteria. Nevertheless, certain points of resemblance can be seen which suggest that a study of the well-established bacteriophage model might throw light on some of the problems of virus infection in man. As the immunity of the lysogenic bacterium is to all intents and purposes permanent, its most probable counterpart in human virus infection is the secure and usually life-long immunity resulting from attacks of certain diseases such as smallpox, yellow fever, measles and poliomyelitis. The solid immunity to further attacks of smallpox enjoyed by a person who has contracted and recovered from this disease is well known. That this immunity may be cellular and not humoral is suggested by the results of vaccination in infants suffering from hereditary hypo-γ globulinaemia in whom the infection may run a normal course (MacCallum, 1959), and also from the experiments of Friedman & Baron (1961) who found that irradiated guinea-pigs, in which no detectable neutralizing antibody could be demonstrated, recovered from vaccinia infection as rapidly as non-irradiated animals, suggesting that production of neutralizing antibody was not necessary for recovery. Long and extensive experience leaves no doubt that a non-fatal attack of yellow fever confers life-long immunity to further infection with the virus. Sawyer (1931) found that the serum of 45 out of 60 subjects who had suffered from yellow fever 30 to 78 years previously—including 5 out of 6 with a 75 years' interval—protected susceptible monkeys from a challenge dose of yellow fever virus. Many of the 45 had no exposure to infection subsequent to the original illness. It is more than unlikely that the antibodies which protected the monkeys had been elaborated during the original attack and had persisted in the circulation for as long as 75 years: their presence clearly indicates some continuing and comparatively recent stimulation of the antibody-producing mechanism, and a possibility which suggests itself is that the susceptible cells (probably liver epithelium) which survived the original infection did so because they were 'lysogenized' by the virus; that this property, incorporated in the infected cells, was passed on to their progeny as a hereditary character; and that the virus which stimulated the antibody-producing mechanism was liberated from one of these lysogenized cells in which, as happens in bacterial cultures, the hypothetical 'provirus' had undergone vegetative development. If this is the explanation, the circulating antibodies may be incidental—they are not to be detected in many who have suffered from yellow fever and so are undoubtedly immune—and the immunity which all recovered cases enjoy may rest fundamentally in the resistant lysogenized cells which in the non-immune

subject would be open to attack by the virus. At least 3 types of poliovirus of different antigenic structure can cause paralytic poliomyelitis, and the accepted practice is to immunize with a vaccine composed of all three. Yet there is a record of an outbreak due to Type I virus being apparently cut short by mass immunization of the community with Type II attenuated virus vaccine (Hale *et al.* 1959). Could this result from a process, analogous to lysogenization of the susceptible cells, giving a cross-immunity similar to that shown by some of the lysogenic Q1 strains? These ideas are speculative, yet the examples given could be amplified and expanded in many ways and still remain so good a fit to the underlying theory that it is speculation which merits careful consideration.

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The Biochemical Mode of Action of the Sulphonamide Drugs*

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It is the first occasion in this series of lectures in which the major part of the problem under discussion concerns the work of the lecturer himself. This is rather like entering the confessional in public, and you will forgive me, I know, for having provided myself with a few lantern slides in order to darken the room from time to time. A difficulty that became very real to me in preparing this lecture was to recall my actual processes of thought at the time of this work 21 years ago. So much has happened since which has a direct bearing on it that it is extremely difficult to cast out things which have since become, so to speak, part of my phenotypic constitution. However, I will try to be strictly honest and if things in fact did not occur in the way in which, in the light of the present knowledge, they ought to have occurred, this is not my fault. I am instructed to discuss the interplay of theory, experiment, models and chance in my work; this means that I really have to be frank!

It is clearly impossible for me to describe my own experiences during the period at issue without telling you something of my own background at the time. The story begins with a very large slice of chance. The problem to be discussed is essentially one of chemical microbiology. I became interested in this subject at 8.30 p.m. on Friday 9 May 1930. At that time the late Dr Marjory Stephenson (a portrait appeared in this *Journal* (1949) **3**, facing p. 329) gave a broadcast talk in a series entitled 'Biochemistry: what it is and what it does'. Her particular topic was 'How microbes live *or* Some aspects of bacterial physiology'. This short talk made a deep impression upon me; I was 18 at the time, which is no doubt an impressionable age. I would like to quote two short passages from it. (I hasten to add this is not due to a phenomenal feat of memory on my part, but to the courtesy and industry of the British Broadcasting Corporation, who after a lapse of nearly 31 years, managed to provide me with a copy of her actual script.) In the course of this broadcast she said:

> The problem of growth lies at the very heart of biochemistry. The biochemist follows the scriptural advice 'consider the lilies how they grow', but all forms of life are lilies to us; when we know how the microbe grows we shall be a long way towards knowing how lilies and lambs grow.

In other words, by studying microbes I would not only learn something of their biochemistry but also of that of all living organisms. This point of view has been amply borne out during the past three decades. Her concluding sentence was:

> I don't know whether I have persuaded any patient listener to think that microbes are an interesting study on their own account, but I can assure you that those of us who spend

* A lecture delivered in the University of Oxford on 17 May 1961 in a series entitled 'Case histories in biological discovery' organized by Dr A. C. Crombie, University Lecturer in the History of Science.

our time trying to persuade these little people to disclose their secrets, find our lives full of interest and often of excitement.

Well, she had at least one convert, for since that broadcast the whole of my scientific life has been devoted either to preparing myself for, or actually studying, the biochemistry of micro-organisms.

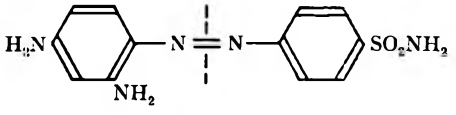
At that time I was in my last year at school and waiting to go up to Cambridge. But in the University of Cambridge was the world-famed School of Biochemistry led and inspired by Gowland Hopkins, and within the Department of Biochemistry worked Dr Marjory Stephenson. So my dreams had perhaps some small hope of materializing. I decided when I went to Cambridge to abandon certain physical sciences, retaining chemistry, but switching over to Botany and Zoology as the other subjects for Part I of the Tripos. Eventually I was able to take up Biochemistry itself for my Part II. When finally the news arrived that I had done well enough to attract a D.S.I.R. grant to stay on and do research I ran straight to Marjory Stephenson who was kind enough to take me on as a Ph.D. student. So you see that my presence here today lecturing on this subject owes much to chance, perhaps fortified by a rather one-track mind.

I spent 6 happy years researching under Marjory Stephenson on problems of bacterial metabolism in a Department which had a very lively atmosphere and where much work on enzymes was in progress and I became fairly knowledgeable, I think, about the dynamic aspects of biochemistry as a whole. In the nineteen-thirties there were two main schools of bacterial chemistry in this country. One led by Marjory Stephenson was mainly concerned with the metabolic aspects, that is the chemical changes induced by bacteria in their environment. The other led by Dr (now Sir Paul) Fildes (a portrait appeared in this *Journal* (1962) **27**, facing p. 335), now happily here with us in alleged retirement in Oxford, studied the nutritional requirements of bacteria, and in particular those vitamin-like substances, which albeit in very low concentrations, were essential for the growth of many bacteria. As we now know many of these have turned out to be precursors of coenzymes or part of the structure of enzymes catalysing key metabolic processes of the cell. Furthermore, the Fildes' school used in their work mainly bacteria of medical interest, whilst we in Cambridge had been concerned with non-pathogens, which are equally important and in nature far greater numerically.

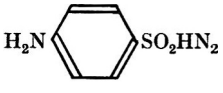
In 1939 Fildes offered me a fellowship to join his team. I accepted this with alacrity not only because it offered an opportunity to extend my experience into the other main current field of bacterial chemistry, but because it had always been Marjory Stephenson's philosophy that problems in our field would be most likely to be solved if attacked at all levels of investigation, which meant of course the nutritional and more biological approaches as well as the more purely metabolic one I had used so far. I joined the Fildes' Unit on the rather inappropriate date of 1 April 1939. I worked for a few months without much success on the nutrition of the gonococcus, an organism which had been shown to require charcoal for best growth. Then we turned to sulphonamides. To recapitulate my own experience up till that time, I was fairly saturated with regard to enzyme and metabolic biochemistry and I was now working in a more biological environment which was also well provided with the know-how of bacterial vitamins.

The sulphonamides: historical background

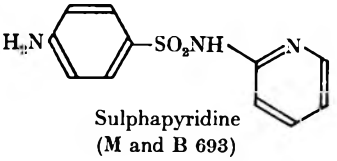
I have time only briefly to sketch the earlier history of the sulphonamides. As long ago as 1885 Paul Ehrlich proposed his view that the organism contained specific receptors which fixed essential foodstuffs, and that toxic drugs might act by fixing themselves to these receptors because of chemical similarity to the foodstuff in question. Amongst such toxic agents were dyestuffs (vital stains) which could clearly be envisaged as becoming fixed by the protoplasm. There was a good deal of work on the bactericidal action of dyestuffs during the first 15 years of this century, notably by Churchman and by Simon & Wood; however, no useful thera-



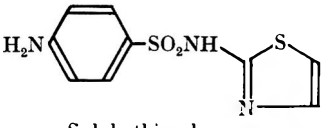
Prontosil rubrum



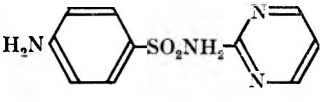
Sulphanilamide



Sulphapyridine
(M and B 693)



Sulphathiazole
(M and B 760)



Sulphadiazine

Fig. 1. Structural formulae of prontosil rubrum and some sulphonamides.

peutic agents emerged. The major step forward was that of Domagk (1935). He was working in the laboratories of I. G. Farbenindustrie where there had been earlier work on the antibacterial action of azo dyes. Domagk found that azo compounds containing a sulphonamide residue had slight activity against streptococcal infections in mice. Following up this clue he showed that a red dye, prontosil rubrum (Fig. 1) had powerful curative effects on streptococcal infections in mice and clinical trials showed it to be highly effective against puerperal fever in humans.

But Domagk had also found that prontosil, although active *in vivo*, had no inhibitory action on the growth of bacteria on culture media in the laboratory. Workers at the Pasteur Institute (Tréfouël, Tréfouël, Nitti & Bovet, 1935) suggested that prontosil was broken down in the animal body, yielding sulphanilamide (Fig. 1) and showed that the latter was active in laboratory cultures. Fuller (1937) finally confirmed that prontosil was indeed broken down *in vivo* to sulphanilamide.

Sulphanilamide led to the discovery of more potent antibacterial chemotherapeutic agents. The molecule was modified chemically by traditional methods, parti-

cularly by workers at May and Baker Ltd. under Dr A. J. Ewins. The most active drugs were those in which a basic substituent was introduced into the amide group producing compounds (Fig. 1) such as sulphapyridine (M and B 693), sulphathiazole (M and B 760), sulphadiazine and so on. Such drugs were being tested by Whitby and his colleagues at the Middlesex Hospital in 1938/9.

Biological activity of sulphonamides: anti-sulphonamide agents

It is perhaps a cliché, though nevertheless in my opinion profoundly true, that biochemical discoveries spring in the main from biological observations. Let us first examine what knowledge had accumulated by the late nineteen-thirties

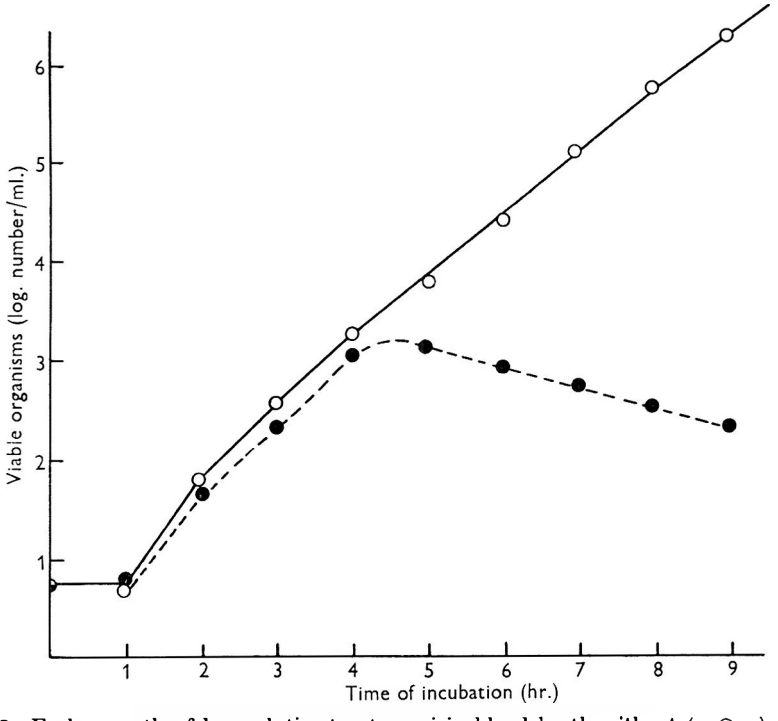


Fig. 2. Early growth of haemolytic streptococci in blood broth without (—○—) and with (—●—) sulphanilamide (10 μM).

regarding the biological nature of sulphonamide action. First, the sulphonamides appeared to be primarily bacteriostatic; they did not kill the bacteria, and living organisms could always be obtained from cultures treated with sulphonamides. A proportion of organisms, however, probably died in artificial culture media when they were prevented from dividing. The evidence was therefore that sulphonamides were not general cell poisons. Secondly, in complex media such as blood broth the organisms underwent a number of divisions in a perfectly normal manner before the effect of sulphonamide became manifest (Fig. 2). The length of this initial period of normal growth depended on the complexity of the medium used, being longest in very complex media (such as the blood broth illustrated). It occurred, however, even if only to the extent of one or two generations, in the simplest medium sup-

porting the growth of the test organism. Thirdly, sulphonamides were relatively ineffective clinically in open wounds where there was considerable pus.

One interpretation of these observations was that material derived from natural sources (such as peptone, blood, etc.) contains substances which in some way prevent the action of the sulphonamides, and furthermore that the bacteria of the inoculum may also contain such substances. Only when they are used up do the sulphonamides act. Early in 1939 McIntosh & Whitby of the Bland-Sutton Institute of the Middlesex Hospital (which also housed the Fildes' Unit) examined and rejected experimentally a number of possibilities for sulphonamide action and by a process of exclusion suggested that they probably act 'by neutralization of some metabolic function or enzymatic activity'. This hypothesis was certainly inspired by informal tea-time discussions with Fildes and his colleagues whose work on growth factor requirements had by then led them to think in terms of essential metabolites, that is vitamin-like substances essential for normal growth and which are synthesized by many bacteria for themselves; they become growth factors for species which cannot achieve their synthesis. In more modern terms of Ehrlich's concepts drugs (including sulphonamides) might act by interfering with the normal function of such essential metabolites.

Clearly, however, whatever the hypothesis, anything that could be found out about the nature of anti-sulphonamide substances present in micro-organisms themselves might throw light on the mechanism of sulphonamide action. This conclusion was apparently reached by several workers more or less simultaneously. Just before our own intensive work on the subject, Stamp (1939) succeeded in extracting from a haemolytic streptococcus material with anti-sulphonamide activity. He did not (with Beall) establish its chemical nature, probably because chemical analysis was attempted at too early a stage of purification. He concluded it was a mixture of substances of relatively low molecular weight. He also suggested that the substance or substances might contain a necessary nutritive factor or coenzyme the production or activity of which is interfered with by the drug. Concurrently with our own work Green (1940) in Sheffield obtained from *Brucella abortus* a partly purified fraction which overcame sulphonamide inhibition of the growth of the same and other organisms; it also greatly stimulated growth. No conclusions were reached at the time about the chemical nature.

This problem was clearly tailor-made for the Fildes' Unit with their wide experience of the detection and isolation of vitamin-like substances for bacteria. Indeed they had recently found glutamine to be an essential growth factor for Streptococcus and in view of the fact that both it and sulphanilamide were amides, had tested whether glutamine might have anti-sulphonamide activity. The results were however negative.

From previous experience the Fildes' group (which I had now joined) took the view that a substance of importance to key metabolic processes in one organism was likely also to be essential to other organisms, and to be of wide distribution. Furthermore, it was by then known that sulphonamides under appropriate conditions inhibited the growth of a fairly wide variety of bacteria. We therefore decided to look for anti-sulphonamide factors in yeast—an organism which had proved in the past a good source of vitamins both for man and bacteria. Since the resources of the Unit were slender for the large scale cultivation of bacteria there was the added

advantage that yeast could be bought by the pound in the baker's shop or begged from the nearest brewery. About the middle of July 1939, Fildes prepared a weakly alkaline extract of yeast (as recommended by Stamp for his streptococcal extracts); it had high anti-sulphonamide activity with the test organism, which was a virulent strain of *Streptococcus haemolyticus*. From then on I took over the work since it was likely to be predominantly biochemical in character.

Before dealing further with technical matters I should like briefly to recall to you the general situation at the time. At the end of July 1939 war appeared to be unavoidable and imminent. Large-scale bombing of cities was expected with consequent high incidence of dirty wounds, delay in the treatment of them and consequent risk of more general infection. At that time, before the birth of penicillin, the sulphonamides were the only successful agents for chemotherapy of bacterial infections. Anything we could do to discover their mode of action might conceivably help us to design other chemotherapeutic agents. The time seemed to be one for urgent work at the bench, rather than for preparing oneself intellectually to attack the problem. I was singularly ignorant of the science of pharmacology, so clearly important for this work, and I had certainly not read all the original work of Ehrlich and other pioneers of chemotherapy. But I was working in an environment experienced in the isolation and study of bacterial growth factors; furthermore, in the laboratory next door Whitby and his colleagues were controlling the clinical tests of the latest sulphonamide products of May & Baker and this provided a sense of reality.

Discovery of the anti-sulphonamide activity of p-*aminobenzoic acid*

It will be clear to you by now that the experimental work on the anti-sulphonamide factor or factors present in yeast was started with the tentative working hypothesis that such a factor might be akin to a bacterial growth factor or vitamin. The technology employed was therefore in general that of research on vitamins. The biological test was fortunately a simple one. The test organism, a virulent strain of *Streptococcus haemolyticus*, was grown on the simplest medium then known to support its growth. This was in fact rather complex and contained peptone, glucose and a number of vitamins known to be essential. To prevent carry-over of anti-sulphonamides the inoculum used was small (about 100 organisms/ml.) Almost full growth was attained in 24 hr. Sufficient sulphanilamide (about M/3000) was added completely to prevent growth for at least 5 days. The yeast extract (or fractions derived from it) was added at falling concentrations each decreasing by a factor of 5. The result of the titration was judged simply by holding the tubes before the window and deciding by visual inspection whether growth had occurred or not. The lowest concentration of the test material giving growth in the presence of sulphonamide before 5 days was considered to be the end point of the titration. I know that in modern terms this technique seems horribly crude, but I do not think any more elaborate method of assessment, such as viable counts or quantitative measurement of turbidity, would have given any more information and would certainly have consumed much more time. I must, however, confess to one stupidity in these tests. The first time I did one the final volume of the medium was in fact 11 ml. instead of the 10 ml. intended. I failed to realize this until almost the end of the work; consequently my concentrations of sulphonamide and other test substances

were all curious values such as M/1100 instead of more usual and sensible M/1000. I can offer no excuse for this lapse.

Two lines of experimental approach were carried out concurrently (Fig. 3). The more biological and kinetic aspects of the anti-sulphonamide action of yeast extracts were examined with relatively crude extracts, and the apparently more important observations repeated with more purified material as it became available. I will return later to this aspect of the work, but it is important for the ultimate results to realize that it neither preceded nor succeeded that about to be described.

The other approach (Fig. 3) was a straightforward attempt to concentrate, purify and isolate the factor itself and establish its chemical nature. It was hoped of course that from the behaviour with the reagents used it would be possible to learn something about the chemical nature of the substance. Furthermore, it was a deliberate policy, before any actual isolation, to try to identify by specific tests some of the chemical groupings in the molecule. I will not attempt any full description of this

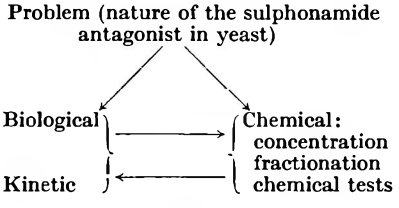


Fig. 3.

search but simply summarize the significant findings. My own first experiment with yeast extract was on 8 August 1939. The methods used were those traditionally associated at the time with the isolation of substances from natural sources. Procedures, for example, such as precipitation of the factor or inactive contaminants with ethanol, acetone and heavy metals, and extraction with a variety of solvents at different pH values. Suffice it to say that some degree of purification was achieved with relatively little loss of the original activity and that the behaviour during fractionation suggested that I was dealing with a single substance (or group of chemically related substances).

One of the main difficulties at the time was the lack of facilities we had for really large scale work, particularly in the first stages of the extraction. To quote one perhaps less serious example, our cold room accommodation consisted only of a domestic type of refrigerator while I frequently had several litres of fluid to store overnight. Eventually I was able to find space for it in the hospital mortuary, hidden away along gloomy corridors in the basement. So my days for several months started and ended with a visit to this cheerless place to rescue or deposit my concentrates from among the white-sheeted figures. So now you know what 'stored in the cold room' may sometimes mean.

Apart from such impediments it is interesting, though perhaps idle, to speculate how much easier the whole thing would have been had modern techniques such as paper chromatography then been available. A few runs on paper with various solvents; detection of the active spots by laying the developed chromatogram on medium containing sulphonamide and seeded with the test organism: we might easily have obtained almost pure material in a few weeks.

However, my material then, though still very crude, seemed sufficiently purified to make it worth while to attempt a few chemical tests. The fact that it was extracted by ether from weakly acid solutions, though not from alkaline, suggested it might be itself a weak acid, perhaps, since it was a natural product, a carboxylic acid. The acidic nature was confirmed by the fact that esterification of the extract with ethanol led to complete loss of activity which was, however, regained when the treated material was hydrolysed:

$$R.COOH + C_2H_5OH \rightleftharpoons R.COOC_2H_5 + H_2O.$$

I also sought evidence as to whether amino groups were present; mild treatment with nitrous acid destroyed all anti-sulphonamide activity, thus suggesting this was possible. Acetylation of the extract with acetic anhydride in pyridine led to complete loss of activity which was regained on hydrolysis:

$$R.NH_2 + CH_3COOH \rightleftharpoons R.NH.OC.CH_3.$$

The material with anti-sulphonamide activity appeared to have both basic and acidic groups. Yet ordinary amino acids were apparently excluded by the fact that the active material was soluble in ether. The most purified material I then had gave a red colour when diazotized and coupled with a naphthylamine derivative. This indicated the presence of an aromatic amino group, though it certainly did not prove that the actual active substance contained such a grouping—merely that it was present in the concentrate, perhaps in the active substance, perhaps only in an impurity.

To summarize then, the evidence indicated at least the possibility that the anti-sulphonamide contained (*a*) a benzene or other aromatic nucleus, (*b*) an amino group and (*c*) a weakly acidic group, possibly a carboxylic group. These chemical properties were also to be observed in sulphanilamide itself (Fig. 1), since it also contains a diazotizable aromatic amino group and the other substituent on the benzene ring (the sulphonamide group, $—SO_2NH_2$) is weakly acidic. I would emphasize that these indications of the possible chemical structure had been deduced not from the properties of an isolated pure substance but from tests with relatively crude material and from the association of biological activity with various chemical properties. It does perhaps illustrate the value of such a combined approach. But clearly the conclusions were only tentative.

I must return now to the more biological and kinetic studies which had been proceeding concurrently, and I will mention only the relevant observations. First, although the initial yeast extracts increased the rate of growth of the test organism on the basal medium, this effect disappeared early during purification, leaving only the anti-sulphonamide action. Such action could not therefore be due to non-specific growth stimulation by the yeast extract. Secondly, quantitative considerations of the relation between dry weight of even the crude extracts and the amount of sulphonamide whose inhibitory action was overcome made it clear that one molecule of the active substance must be antagonizing the action of many molecules of sulphanilamide. Thus it was unlikely that the antagonist was inactivating the sulphonamide by combining with it in some way. Finally, and most important, there was a rather strict quantitative relationship between the concentration of the drug and the amount of yeast extract required to overcome its action. If the drug

were increased 5- or 25-fold then the amount of yeast extract required to restore growth was similarly increased.

Taking together the main results of the two methods of approach (Fig. 3) there was evidence that the antagonist might be similar in chemical structure to sulphanilamide itself and that there was a strict quantitative relationship between the two in controlling growth. To me, with the background of my training in Cambridge, this situation was clearly reminiscent of the phenomenon of competitive inhibition of enzyme reactions by substances analogous to the substrate. This phenomenon had first been discovered by Quastel & Wooldridge (1928) in Hopkins's laboratory for the succinic dehydrogenase of *Escherichia coli*. This enzyme, which catalyses the oxidation of succinate to fumarate in the presence of a hydrogen acceptor, was competitively inhibited by malonate, which is the analogue of succinate with one less CH_2 group (Fig. 4); when malonate concentration is increased, the inhibition of enzyme activity can be overcome by increasing the concentration of succinate to a similar extent. In the present case one would have to suppose the yeast factor to be the substrate of a bacterial enzyme and sulphanilamide the analogue which competitively inhibits the activity of the enzyme.

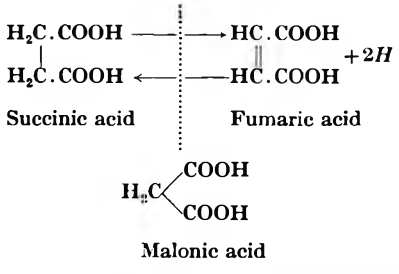


Fig. 4. Competitive inhibition of succinic dehydrogenase.

I was encouraged by this reasoning to try a few shots in the dark and to test for anti-sulphonamide activity some compounds which were both similar in structure to sulphanilamide and compatible with the sort of chemistry deduced for the yeast extract factor. One compound was, from what I have said earlier, an obvious candidate for the first test. It was *p*-aminobenzoic acid and was fortunately obtainable at small cost from the British Drug Houses Ltd.; the test was put up at 1 p.m. on 14 December 1939 and I must admit that 12.30 p.m. on 15 December, when I took the tubes out of the incubator, was (and still is) the most exciting moment in my scientific life. The photograph (Pl. 1, fig. 1) of a recent repetition of the important parts of this experiment perhaps also illustrates the simplicity of the test procedure. A different organism had to be used since the original was no longer available and to make things fairer I asked my colleague Dr J. G. Morris to put up the test; at any rate it shows that the critical findings can still be repeated. There are controls on the basal medium without (tube 1) and with (tube 2) sulphanilamide showing growth and no growth respectively. A control with *p*-aminobenzoic acid only (tube 5) shows that it does not itself inhibit growth. Finally, tubes (3 and 4) to which both sulphonamide and *p*-aminobenzoic acid have been added show that the inhibitory effect of the former has been overcome. A repeat of the original experiment was in the incubator by 3.45 p.m. on the same day; how-

ever, all was well and the *p*-aminobenzoic acid was so active that it took me two further tests to reach the end point of the titration.

As with the yeast extract there was a clear-cut competitive and quantitative relationship between the concentration of sulphanilamide and the concentration of *p*-aminobenzoic acid required to overcome its inhibition. (This is illustrated for another organism in Fig. 8.) One molecule of *p*-aminobenzoic acid antagonized the action of about 10^4 molecules of sulphanilamide. It was highly specific in its action; the *ortho* and *meta* isomers were inactive, and any weak activity of related substances was probably explained by their conversion to *p*-aminobenzoic acid itself or by the presence of the latter as an impurity.

There was thus strong evidence from the close correspondence of biological and chemical properties that the active factor in yeast extract was *p*-aminobenzoic acid though in the absence of actual isolation this was not certain; it might have been a closely related compound, though none available for test had comparable activity.

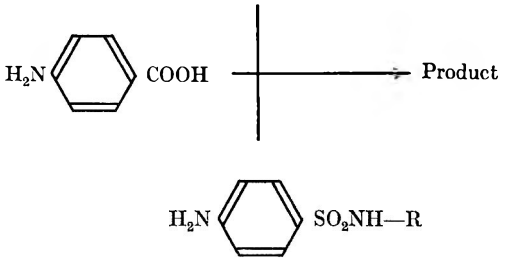


Fig. 5. Competitive inhibition of the utilization of *p*-aminobenzoic acid by sulphonamides.

The hypothesis

In the light of these results I put forward a working hypothesis for the biochemical mode of action of the sulphonamides (Woods, 1940). In its simplest terms this suggested that *p*-aminobenzoic acid was a substance essential for the growth of bacteria, but was normally synthesized by them for themselves; it belonged to the class which Fildes had christened essential metabolites. It was further suggested that the sulphonamides, by virtue of their similarity in chemical structure to *p*-aminobenzoic acid, competitively inhibit the enzyme reaction by which the latter is further utilized by the organism (Fig. 5). In enzymological terms the sulphonamide was envisaged as competing with *p*-aminobenzoic acid for combination with the active centres of the enzymes concerned.

My brief for this lecture says that I should assess the role played by models in the work. I have to confess that in framing this hypothesis originally I did not build molecular models of the substances in question, but relied only in a simple-minded way on one-dimensional geographical chemistry, that is on the structural formulae as you see them in Fig. 5. However, the resemblance in molecular architecture between *p*-aminobenzoic acid and sulphanilamide may be seen in the photographs of the molecular modes (Pl. 1, fig. 2). Of course it is not only the spatial distances between polar groups which may combine with the enzyme that matters, but also the charge on such groups.

This work and the inferences drawn from it was first reported at a meeting of the

Biochemical Society on Saturday 17 February 1940 in Sheffield (Woods & Fildes, 1940). It was the most interesting piece of work in which I have been concerned and certainly the most rapid. In retrospect it scarcely seems possible that it all happened in just over 6 months; we must have had a lot of luck.

I worked on for some time examining the specificity of the *p*-aminobenzoic acid/sulphonamide relationship and found it to hold with every sulphonamide drug and with every organism I tested. I also continued attempts to isolate the material from yeast in pure, crystalline form but did not get beyond about a few milligrams of about 50% pure product. By the next summer I had been removed to other activities more directly concerned with the war and was able to take no further part, greatly to my regret, in the immediate development of the work and the further testing of the hypothesis.

Confirmation of the hypothesis

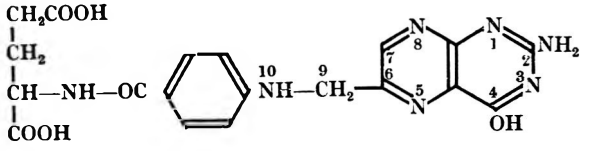
I should like, however, to give some brief consideration to the extent to which the hypothesis has been confirmed. It predicted that *p*-aminobenzoic acid was a key substance in bacterial metabolism, that it was in fact an essential metabolite. At the time it was not known to be a growth factor for any organism, nor was it even known to be of biological occurrence. The best confirmation the hypothesis could receive was therefore an observation that *p*-aminobenzoic acid was an essential growth factor for some bacterium. This was not long in coming. Rubbo & Gillespie (1940) in Australia had been pursuing an unknown growth factor for *Clostridium acetobutylicum* which was present in yeast concentrates. In the light of our prediction they tried *p*-aminobenzoic acid and found it highly active; this was announced in a letter to *Nature* published on 28 December 1940. They also succeeded in isolating a small amount of the benzoyl derivative of *p*-aminobenzoic acid from yeast; a year later Blanchard (1941) in the U.S.A. isolated the pure substance itself and in better yield. Since then it has been found to be essential for the growth of a wide variety of bacteria and other micro-organisms embracing many families and genera and including induced or biochemical mutants of both moulds and bacteria. I shall not bore you with a list—there must be quite fifty of them. Organisms which do not require it as a growth factor have been shown to synthesize it for themselves since extracts promote the growth of organisms which do require an exogenous source. *p*-Aminobenzoic acid has been proved therefore to be of natural occurrence and to be an essential metabolite in the full sense of the term.

p-Aminobenzoic acid has also been reported as having a number of biological effects or vitamin-like activities with the so-called higher forms of life. It was said to be a growth factor for the chick and to increase fertility and lactation in rats. Probably some of these effects may be explained by its conversion to folic acid (see later) by microbes in the gut. There was a number of papers, not always with agreeing results, suggesting that it restored hair colour both in experimental animals and man. One worker (Sieve, 1941) took fifty hospital patients at random ranging in age from 21 to 55 years with greying hair and observed, after 2 months' feeding with *p*-aminobenzoic acid, marked darkening in all cases. Possibly as a result of such work my vitamin was finally featured in a fashion magazine for ladies.

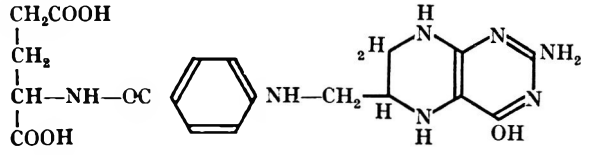
A number of other workers besides myself investigated the general applicability of the relationship between *p*-aminobenzoic acid and sulphonamides and found it to

hold with virtually every combination of different sulphonamide and different organism tested. This again provides general support for the hypothesis, since from other experience one would expect an essential metabolite to have metabolic importance in all micro-organisms.

One point had been puzzling from the outset. Sulphonamides which are intrinsically more active than sulphanilamide itself (such as sulphathiazole, Fig. 1) appear to be chemically less like the metabolite than is sulphanilamide. However, satisfactory physico-chemical explanations for this have come from the work of Bell & Roblin (1942) and others. It appears that the introduction of a weakly basic



(*a*)



(*b*)

Fig. 6. (*a*) Folic acid (pteroylglutamic acid) and (*b*) tetrahydrofolic acid (tetrahydropteroylglutamic acid).

substituent into the $—SO_2NH_2$ group renders the electrical properties of the modified group ($—SO_2NHR$), as judged by ionization and dissociation, even more nearly akin to the properties of the carboxylic acid group of *p*-aminobenzoic acid.

The hypothesis also predicted that sulphonamides inhibited the further utilization of *p*-aminobenzoic acid by the organism. What then is the nature of this reaction (Fig. 5) and especially of its product, which, if the theory is correct, must be of equal importance to the organism? Work during the war years in the U.S.A. on unknown vitamins or growth factors for birds, monkeys and several bacteria led to the realization that these factors were the same or similar. It culminated in the isolation, degradation and synthesis of the factor for *Lactobacillus casei* by the workers at Lederle Laboratories (Angier *et al.* 1946). This factor, called folic acid (Fig. 6), because it was first studied in extracts of spinach leaves, has a central *p*-aminobenzoic acid residue, linked through its carboxylic group to glutamic acid and through its amino group and a methylene bridge to a pteridine ring structure. (The pteridines had first been described as of natural importance in the pigments of butterfly wings by Gowland Hopkins.) There has turned out to be a whole family of folic acids with different activities for different test organisms, but all contain the *p*-aminobenzoic acid and pteridine residues. To cut a long story short, it has now become clear that the substance with ultimate biological activity in the cell (as a

coenzyme) is a derivative with a reduced (tetrahydro) pteridine ring (Fig. 6) and probably with two further glutamic acid residues in γ peptide linkage with the first glutamic acid and with one another.

The cellular function of folic acid both in bacteria and higher forms is as a coenzyme in enzyme systems for the transport of units containing a single carbon atom from one molecule to another (see, for example, the review by Rabinowitz, 1960). It can do this with the carbon atom at either of two oxidation levels, that is either formyl (—CHO) or hydroxymethyl (—CH_2OH). The one-carbon residue released from the

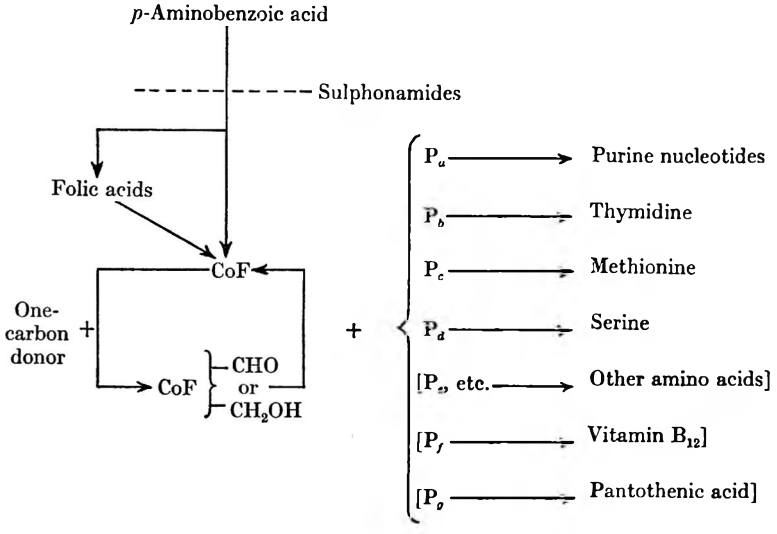


Fig. 7. Diagram summarizing the cellular functions of *p*-aminobenzoic acid and folic acid. CoF is the coenzyme form of folic acid; P_a etc., are precursors of ultimate products. [ ], reaction indicated in only one or a few organisms. (From Woods & Tucker, 1956.)

donor becomes combined with the N^{10} atom (of the *p*-aminobenzoic acid residue) or forms a ring between the N^{10} and the N^5 (Fig. 6). This compound reacts with an acceptor and transfers the one-carbon residue to it (Fig. 7).

Such one-carbon transfer reactions are essential steps in the synthesis of several amino acids, purines and thymine (the pyrimidine unique to DNA); the situation is summarized schematically in Fig. 7). Since inhibition of the metabolism of *p*-aminobenzoic acid creates a shortage of the folic acid coenzyme and thus in turn of essential constituents of both protein and nucleic acid, it is easy to see why sulphonamides have such a drastic effect on growth. Sulphonamide inhibition can also be overcome by appropriate mixtures of amino acids, purines and thymine; one is then adding preformed the ultimate products of the inhibited reaction. Such observations have indeed provided key clues for the elucidation of the function of folic acid.

There is now ample and convincing evidence that sulphonamides do in fact inhibit the conversion of *p*-aminobenzoic acid to folic acid. However, the precise biochemical mechanism of this conversion and the nature of the intermediates is not yet known with certainty, though there is recent progress suggesting that the folic acids with the reduced pteridine nucleus are formed directly. It must be confessed,

however, that after 21 years I still cannot tell you the exact nature of the enzyme reaction which has *p*-aminobenzoic acid as substrate and is inhibited by sulphonamides. This I think is the only notable gap now in the story.*

If utilization for the synthesis of folic acid is the main or sole cellular function of *p*-aminobenzoic acid, the original hypothesis would demand two things. First, folic acid should also overcome sulphonamide inhibition. Secondly, it should do so irrespective of the concentration of sulphonamide; the relation should not in this case be a competitive one. This would be expected because folic acid would be the product of the blocked reaction and beyond the point of attack of sulphonamides; provided sufficient folic acid were given for the minimum needs of the organism (and provided it can get into the organism) growth should occur irrespective of sulphonamide concentration. These expectations have been fulfilled with a number of organisms tested. I will quote one example only from our own post-war work (Woods, 1954); this concerns the anaerobe *Clostridium tetanomorphum* which requires *p*-aminobenzoic acid for growth. There is the usual straight line competitive relation between *p*-aminobenzoic acid and sulphanilamide over a 1000-fold range (Fig. 8). Folic acid (two forms were tested) was slightly more active than *p*-aminobenzoic acid in supporting growth in the absence of sulphonamide. However, once this amount of folic acid was present it was sufficient to support growth with any concentration of sulphanilamide (Fig. 8).

But with other bacteria folic acid, in the forms so far tested, is without effect either in replacing *p*-aminobenzoic acid or overcoming sulphonamide inhibition. Such organisms may be impermeable to folic acid (though this is unlikely) or unable to convert the form supplied to what we now believe to be the ultimate coenzyme form. The organisms which do not respond to folic acid include all those normally attacked by sulphonamide therapy in man. Whatever the reason for it this inactivity of folic acid may be of critical importance in explaining the therapeutic success of the sulphonamides.

Sulphonamides, like all other chemotherapeutic agents, are only successful because they are selectively toxic to the microbe and relatively harmless to the host. Any theory of sulphonamide action must explain this. How far can we go on the basis of our original hypothesis and the later work which I have described and which confirms it? I think the basis of a reasonable explanation is there.

Folic acid is an essential nutrient or vitamin for the animal and must be provided preformed in the diet. It follows therefore that animal cells cannot synthesize it. The metabolic lesion induced in sensitive bacteria by sulphonamides already exists in animals, and sulphonamides therefore can have no effect on them. It is of importance in this connexion that bacteria which require preformed folic acid for growth are also totally insensitive to sulphonamides. One must ask, however, why the folic acid present in host tissues does not overcome the action of the sulphonamides on the bacteria. I have already indicated that forms of folic acid so far tried, which include the forms in which it appears to occur free in animal tissues, are ineffective

* This gap has now been filled. Since this lecture was delivered Brown (1962) has shown that sulphathiazole and other sulphonamides inhibit competitively the activity of cell-free extracts of *Escherichia coli* catalysing the condensation of *p*-aminobenzoic acid with 2-amino-4-hydroxy-6-hydroxymethyldihydropteridine to form dihydropteroic acid; the product is further transformed to tetrahydropteroylglutamic acid (Fig. 6).

with many organisms including those which can be attacked by sulphonamide therapy. It is possible that the ultimate functional coenzyme form of folic acid only exists in cells firmly bound to or combined with the enzymes of which it is a part.

The idea that drugs might act by competing with substrates of enzyme systems was clearly not a new one and did not emerge as a result of the present work. However, the successful explanation of the action of a major group of chemotherapeutic agents along these lines certainly appeared to stimulate a lot of thought and experi-

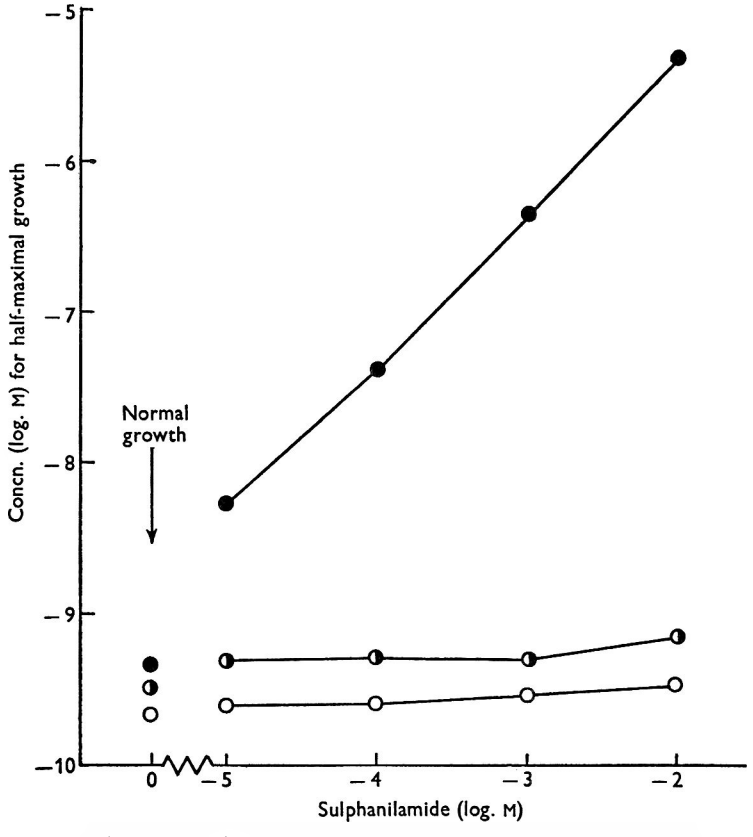


Fig. 8. The requirement of *Clostridium tetanomorphum* for *p*-aminobenzoic acid or folic acid for growth in the presence of varying concentrations of sulphanilamide. *p*-Aminobenzoic acid, —●—; pteroylglutamic acid, —○—; N^5-formyltetrahydropteroylglutamic acid (leucovorin), —◑—. Data from unpublished experiments of Woods & Ballio. (From Woods, 1954.)

ment. Vast numbers of analogues of other essential metabolites were synthesized and examined both for antibacterial and anti-cancer activity. A great many substances with antibacterial activity emerged and some have been very useful tools in metabolic studies as specific inhibitors. I do not think that any really successful therapeutic agent of the calibre of the sulphonamides has emerged.

Finally, I should like again to emphasize how much the outcome of this work owes to my own two teachers Dr Marjory Stephenson and Sir Paul Fildes. Of both of them it may truly be said, and with some appropriateness, that 'infection, not instruction, is the secret of education'.

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EXPLANATION OF PLATE 1

Fig. 1. Reconstruction of an early experiment showing antagonism by *p*-aminobenzoic acid of inhibition of growth by sulphanilamide. 1, Control; 2, +sulphanilamide; 3 and 4, +sulphanilamide and *p*-aminobenzoic acid; 5, +*p*-aminobenzoic acid only.

Fig. 2. Molecular models of *p*-aminobenzoic acid (left) and sulphanilamide (right).

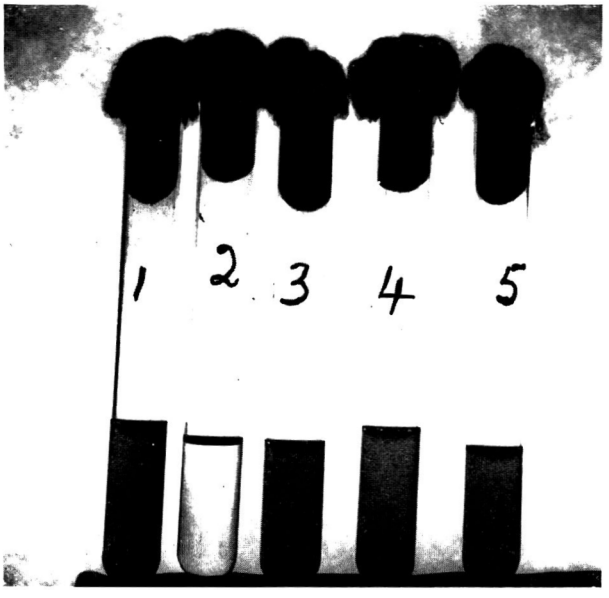

Fig. 1

Fig. 2

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Foamy Virus of Monkeys

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SUMMARY

Foamy virus has occurred in 40–60% of vervet, rhesus and cynomolgus monkey kidney tissue cultures, but has been absent from *Erythrocebus patas* cultures. Factors influencing its passage in tissue culture have been investigated. The virus is sensitive to ether and chloroform. Ultracentrifugation indicated a particle size of greater than 70 mμ. No haemagglutination or haemadsorption could be demonstrated with erythrocytes from a variety of animals. No reduction in titre resulted from eleven months' storage of a serum-free suspension at $-20°$. Stability at other temperatures was determined.

INTRODUCTION

A cytopathic effect, commonly occurring spontaneously in rhesus monkey kidney tissue cultures and characterized by the formation of syncytia which developed a 'foamy' or 'lacey' appearance, was described by Rustigian, Johnston & Reihart in 1955. Brown (1957) and Ruckle (1958) showed that the foamy cytopathic effect was transmissible in rabbit kidney cultures, Brown also demonstrating that the transmissible agent was filtrable. Ruckle indicated a superficial similarity between the cytopathic effects of the foamy agent and the measles-like MINIA (monkey intranuclear inclusion agent). Johnston (1961) reported the isolation of foamy agent from the throats of monkeys. He also isolated, from *Macaca cyclopsis*, an agent causing a cytopathic effect in rabbit kidney cultures identical with that of the strains of Rustigian *et al.* (1955), but which was serologically distinct from these strains. Malherbe & Harwin (1957) described an agent, termed SA 1, which caused a foamy-type cytopathic effect in kidney cultures from vervet monkeys (*Cercopithecus* sp.). In 1957 Hull & Minner stated that their many attempts to subculture foamy agent in monkey kidney tissue cultures had failed; they did not therefore include it in their classification of monkey viruses.

This paper details further properties of this agent and factors influencing its passage in tissue culture.

METHODS

Viruses. The foamy virus strain investigated was isolated from a cynomolgus kidney culture by subculture to rabbit kidney tissue. A large pool was prepared by a further subculture into rabbit kidney tissue and stored at $-20°$. SA 1 was kindly supplied by Dr H. Malherbe, South African Institute for Medical Research, Johannesburg, and FV 21 by Dr P. Johnston of The Jefferson Medical College of Philadelphia. Several strains of SV 5 and MINIA which occurred spontaneously in kidney cultures of various monkey species formed the basis of observations made in this paper.

Tissue cultures. Monolayer monkey kidney and rabbit kidney cultures were used, the initiating medium consisting of Earle's balanced salt solution, 0·5% lactalbumin hydrolysate, 2·5% calf serum (5% for rabbit cultures), 0·22% sodium bicarbonate and 100 units/ml. each of penicillin and streptomycin. The maintenance medium for monkey cultures was Earle's salt solution, 10% liver digest (Smith, 1961), 0·22% sodium bicarbonate, penicillin and streptomycin, and for rabbit cultures, Earle's salt solution, 5% calf serum, 1% lactalbumin hydrolysate, 0·22% sodium bicarbonate, penicillin and streptomycin. Tube cultures were rolled at 37°.

Titrations. Serial dilutions of 0·5 $\log_{10}$ steps were inoculated into rabbit or patas tube cultures, 0·5 ml. per tube. The cultures were examined microscopically after 7 or 8 days. Titres were deduced by the Kärber equation and are expressed as $\log_{10}$ TCID50/0·5 ml.

Neutralization tests. Each of a series of doubling dilutions of serum was mixed with an equal volume of virus suspension (titre 2·5). After one and a half hr. incubation at 37° each reaction mixture was inoculated into rabbit or patas tube cultures, 0·5 ml./tube, and examined 7 or 8 days later.

Diethyl ether sensitivity. Equal quantities of virus suspension and diethyl ether were shaken together and held at 4° for 18 hr. The ether was evaporated under reduced pressure.

Size estimation by ultracentrifugation. A virus suspension was centrifuged for fifteen minutes in the SW39 rotor at 39,000 r.p.m. (125,000 *g*) in a Spinco model L. The centrifuge tube contained 2·5 ml. of 3% agar, above which was 2·5 ml. virus suspension. Pre- and post-centrifugation samples were titrated.

Stability and heat sensitivity. One ml. volumes of foamy virus suspended in serum-free rabbit kidney tissue culture fluid clarified by centrifugation were sealed in 2 ml. glass ampoules and held at the appropriate temperatures. After exposure to 45° or 50° the ampoules were immediately cooled in ice water.

Haemagglutination and haemadsorption tests. Haemagglutination tests were done in plastic agglutination plates by mixing equal volumes of virus suspension and 0·25% suspensions of washed erythrocytes in veronal buffered saline, and incubating at the appropriate temperature. Tissue cultures showing cytopathic effect were tested for the haemadsorptive property by inoculating washed erythrocytes into the culture fluid to produce an approximately 0·5% suspension. The cultures were examined microscopically after allowing 1 hr. for the erythrocytes to settle onto the cell sheet.

Hyperimmune sera. An antiserum to the foamy virus was prepared in rabbits by a series of six intramuscular inoculations, the first consisting of 3 ml. of virus (titre 2·7) homogenized with an equal quantity of a mixture of nine parts oil (Esso grade 23, technical white) and one part sorbitan mono-oleate. Each of the remaining inocula was of 3 ml. of virus only. The measles antiserum was prepared in the same manner, the Edmonston strain of virus being used. The SA1 antiserum was provided by Dr H. Malherbe.

RESULTS

Comparison of the cytopathic effects of foamy virus, SV 5 *and* MINIA *in monkey kidney cultures*

The 'spontaneous' occurrence of foamy virus in normal, uninoculated primary cultures was characterized by the formation of syncytia, which tended to be irregular in shape and contain clusters of nuclei. The lesions usually appeared 9–21 days following the preparation of the cultures. No inclusion bodies have been observed. Vacuolation of the syncytium usually took place producing the characteristic 'foamy' appearance. Plate 1, figs. 1, and 2, show the characteristic lesions unstained and stained by the haematoxylin eosin technique.

The presence of SV 5 in primary monkey kidney cultures has not usually produced a cytopathic effect. On subculture into monkey kidney culture a cytopathic effect could be produced, involving the formation of syncytia which soon lost their syncytial nature to become masses of cytoplasm containing small vacuoles and remains of nuclei (Pl. 2, fig. 3). The cytopathic effect was pronounced about 3 days following subculture.

In some tissue culture batches the cytopathic effect produced by foamy virus has been rather similar to that of SV 5. In such cases the foamy syncytia tended to roll up and leave a hole in the tissue sheet surrounded by 'spongy' masses of cytoplasm (Pl. 3, fig. 4). The lesions shown in Pl. 1, fig. 1, Pl. 3, fig. 4, were caused by the same strain of foamy virus; the tissue cultures, however, came from different rhesus monkeys.

Several isolates of MINIA, neutralizable by measles antiserum, were examined and found to have a cytopathic effect readily distinguishable from that of foamy virus. Spontaneous lesions in primary cultures took the form of rounded syncytia surrounded by swollen cells. The nuclei of a syncytium were arranged in a complete or broken ring, the cytoplasm within which was more refractile than that without (Pl. 4, fig. 5). Haematoxylin eosin staining showed a large eosinophilic inclusion body within each nucleus; the dense cytoplasm within the circle of nuclei was also eosinophilic (Pl. 4, fig. 6).

Incidence of foamy virus in kidney tissue cultures of different monkey species

The percentage of cultures, from four different monkey species, showing the foamy effect, together with the geographical region from which the monkeys originated, are shown in Table 1.

Growth and transmission in tissue culture

The growth of foamy virus in a Roux bottle rhesus kidney culture, inoculated with 50 TCID 50/0·5 ml. of culture fluid, was followed by titrating samples removed serially; the values are plotted in Fig. 1. At no stage following virus inoculation was the culture medium changed. The foamy cytopathic effect which appeared progressed to destroy the whole cell sheet. The virus titres reached in cultures experimentally infected with foamy virus have not been found to exceed 3·5.

The progress and spread of the cytopathic effect following experimental infection of a cell sheet was found to depend on the quantity of virus inoculated. The cytopathic effect produced by inocula of greater than about 5 to 10 TCID 50/tube culture usually progressed to destroy the cell sheet. If only one or a few TCID 50 were

inoculated a proportionately small number of lesions were formed, which did not spread or multiply in number. Both progressive and non-progressive effects could be observed in foamy virus titrations in monkey kidney cultures. Spontaneous foamy lesions which did not progress or multiply have been observed in primary monkey kidney cultures. It has not usually been possible, by subculture into rabbit or monkey kidney cultures, to detect foamy virus in cultures showing only a few non-progressive lesions.

The sensitivity of unaffected tissue in cultures containing only a few lesions to superinfection with foamy virus was investigated as follows. Patas kidney tube cultures were inoculated with foamy virus resulting in the development of about

Table 1. *Incidence of foamy virus in kidney cultures*

Monkey	Region of origin	% of cultures foaming
Macaca mulatta (rhesus)	India	46% of 1615 cultures
Macaca irus (cynomolgus)	Thailand	65 % of 259 cultures
Cercopithecus pygerythrus centralis (vervet)	Kenya and Somalia	40 % of 65 cultures
Erythrocebus patas (patas)	Nigeria	0 % of 296 cultures

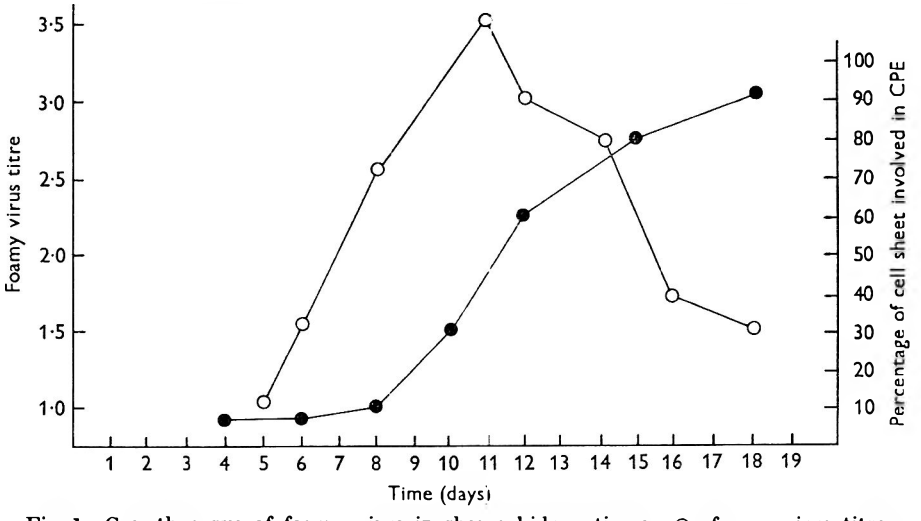


Fig. 1. Growth curve of foamy virus in rhesus kidney tissue. ○, foamy virus titre; ●, % of cell sheet invoved in cytopathic effect (CPE).

four foamy lesions per culture six days later. At the time of development of these lesions a third of the cultures was infected with a further 30 TCID50 per culture. Three days later another third of the cultures was similarly inoculated. Five days following the second superinfection all the cultures were examined. Those which had not been superinfected remained unchanged in appearance, having the same number of lesions as 8 days previously. All superinfected cultures showed a marked increase in the number of foamy lesions.

The readiness with which foamy virus could be subcultured was found to be dependent on the species of tissue cultures involved. The cytopathic effect produced by subculture from monkey kidney to monkey kidney was considerably less pronounced than that produced by either subculture from monkey kidney to rabbit

kidney or rabbit kidney to monkey kidney. Whereas samples of foamy virus grown in monkey kidney cultures produced no cytopathic effect when titrated in the same tissue, titration in rabbit kidney cultures revealed titres of 2·5–3·0. The degree of cytopathic effect produced by subculturing from rabbit kidney to monkey kidney was found to vary with the species of monkey kidney involved. The most marked effects were obtained with kidney cultures from *Erythrocebus patas*.

Antigenic relationship between foamy viruses

A rabbit immune serum prepared against the cynomolgus foamy isolate described in this paper had a neutralization titre of 1/500–1/1000. The same titre was obtained against the SA1 virus of Malherbe & Harwin (1957) and a type 1 foamy virus (FV21) of Johnston (1961). Rabbit antiserum against SA1 neutralized both SA1 and the cynomolgus foamy isolate to a titre of 1/50. Foamy virus strains from five cynomolgus, six vervet and two rhesus kidney cultures were all neutralized by the antiserum against the cynomolgus isolate to the same titre as the homologous virus. These results indicate that all the above strains are antigenically similar and correspond to Johnston's serologic type 1.

Physical properties

Ether sensitivity. Eighteen hr. at 4° treatment with diethyl ether reduced the titre from 2·9 to $<0·5$. Treatment with chloroform and fluorocarbon ($Cl_3F_3C_2$) under similar conditions also destroyed the viability.

Size. Ultracentrifugation (125,000 *g* for 15 min.) reduced the titre of a clarified culture fluid suspension from 2·75 to 1·5. Concentration of foamy virus suspensions at higher speeds indicated that the viability is not destroyed or reduced by ultracentrifugation, the increase in titre of the pellet corresponding to the reduction in titre of the supernatant. Comparison of these results with the sedimentation behaviour of other viruses in the swinging bucket rotor of the Spinco ultracentrifuge indicates a particle diameter of greater than 70 mμ. This conclusion is only valid if foamy virus is non-filamentous, and has a hydrated particle density similar to other viruses. Attempts to study the particle by electron microscopy of phosphotungstate negatively contrasted concentrates were not successful due to the difficulty in obtaining satisfactory concentrates from a virus producing such low titres in tissue culture fluids.

Stability and heat sensitivity. The stability at various temperatures is summarized in Table 2.

Haemagglutination and haemadsorption. No haemagglutination or haemadsorption could be detected at 4°, 22° or 37°, using erythrocytes from the following animals: rhesus, cynomolgus, vervet, patas, Sykes and Papio monkeys, human, horse, dog, cat, ferret, rat, rabbit, guinea pig or chicken. Concentrated foamy virus with a titre of 5·0 did not agglutinate guinea pig erythrocytes at 4°, 22° or 37°.

DISCUSSION

The freedom of *Erythrocebus patas* kidney cultures from spontaneous foamy virus, and the clarity of the cytopathic effect produced by the inoculation into such cultures of foamy virus grown in rabbit tissue, considerably facilitated the study of this virus.

The difficulties that are associated with the subculture and transmission of foamy virus, particularly in monkey kidney cultures, may be explained by the observations made in this paper. The growth curve demonstrates that low titres only are reached. Less than 10 TCID50/0·5 ml. of culture fluid were detectable at 4 days following experimental inoculation, even though a clear cytopathic effect had appeared. The decrease in titre following the maximum of 3·5 probably resulted from less virus being released from the tissue combined with the relative lability of the virus at 37° (Table 2). Fluid subcultured early or late from such a foamy infected tissue would contain only a small number of tissue infective doses.

The small, isolated foamy lesions occurring in monkey cultures, either spontaneously or from experimental infection, have usually been non-progressive, the major part of the cell sheet remaining unaffected, even though its sensitivity to superinfection by foamy virus was retained. This lack of spread is apparently due to

Table 2. *Stability at various temperatures of foamy virus suspended in a serum-free medium*

Temperature	Titre at commencement of experiment	Titre after time intervals indicated below								
		2·75	2·5	2·25	2·0	1·75	1·5	1·25	1·0	< 1·0
−20°	2·75	48 weeks	—	—	—	—	—	—	—	—
4°	2·75	—	—	12 weeks	—	20 weeks	—	—	—	29 weeks
22°	2·5	—	—	4 days	6 days	8 days	—	—	—	—
37°	2·75	—	—	1 day	—	2 days	—	—	—	3 days
45°	2·75	—	—	—	15 min.	30 min.	—	40 min.	60 min.	90 min.
50°	2·0	—	—	—	—	—	—	—	—	15 min.

less than one TCID50 being released into the culture fluid. 'Auto-inhibition' probably does not occur for, although it is sensitive to interferon, foamy virus does not stimulate interferon production (Plummer, to be published).

The poor development, or complete absence, of the lesions when subculturing from monkey tissue to monkey tissue, undoubtedly contributes significantly to the difficulties of tissue culture passage. Added to this is the resistance to foamy virus of any cultures containing SV 5, due to the production of interferon by SV 5 and the sensitivity to interferon of foamy virus (Plummer, to be published). This emphasizes the necessity for screening by the haemadsorption technique all monkey kidney cultures to be used for passaging foamy virus, particularly in view of the presence of SV 5 in primary cultures frequently being unaccompanied by a cytopathic effect.

Classification of the virus is not yet possible. However, it can be excluded from the ether resistant groups, and lack of inclusion bodies excludes it from the 'nita-viruses' (Andrews *et al.* 1961). It cannot be placed amongst the Myxoviruses if haemagglutination is to remain a criterion for inclusion in this group. Work on the structure of the virus clearly needs to be done to assist in its classification.

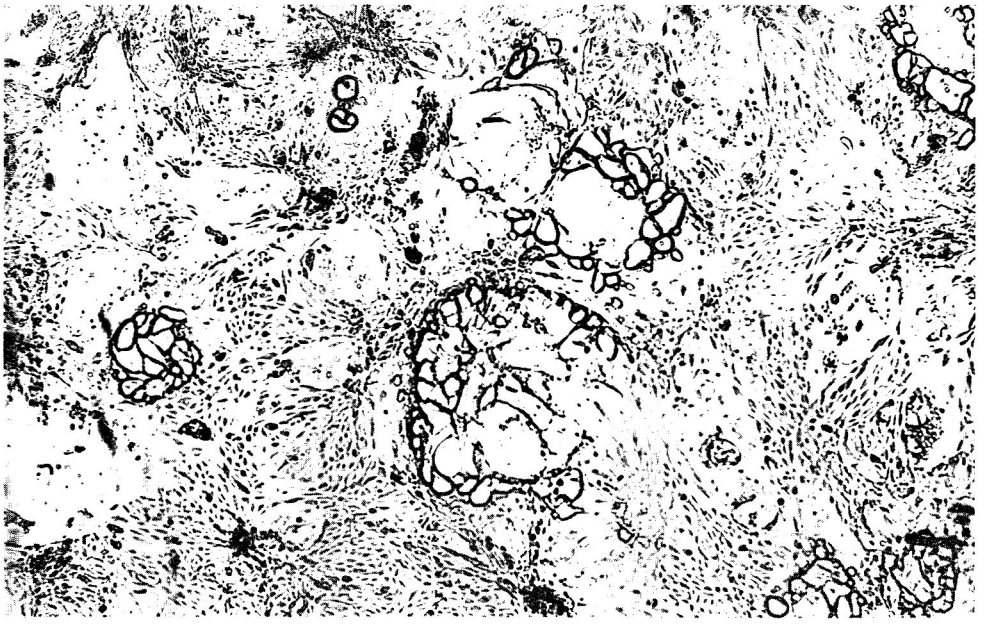


Fig. 1

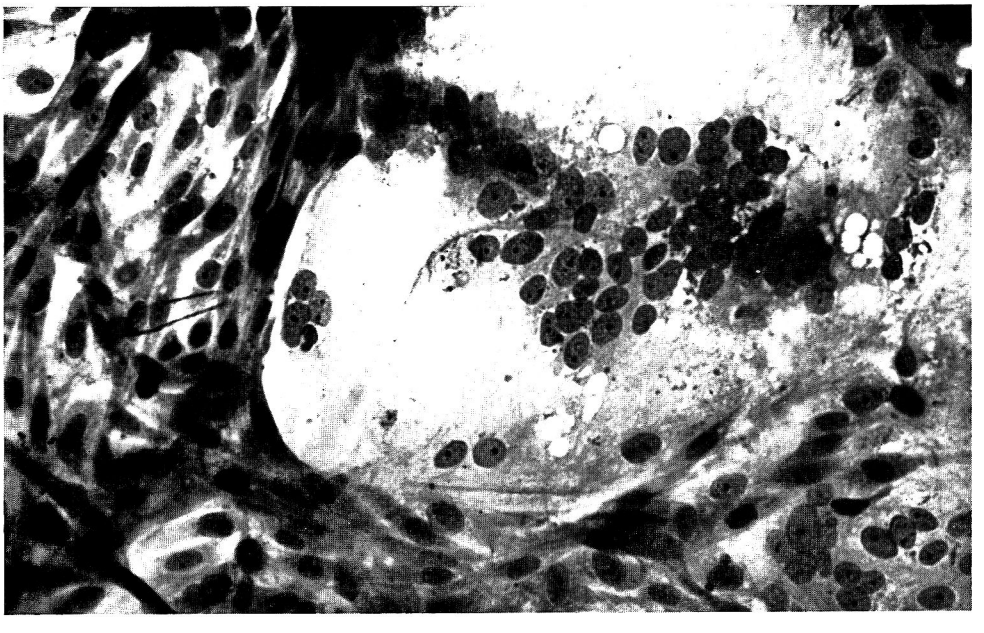
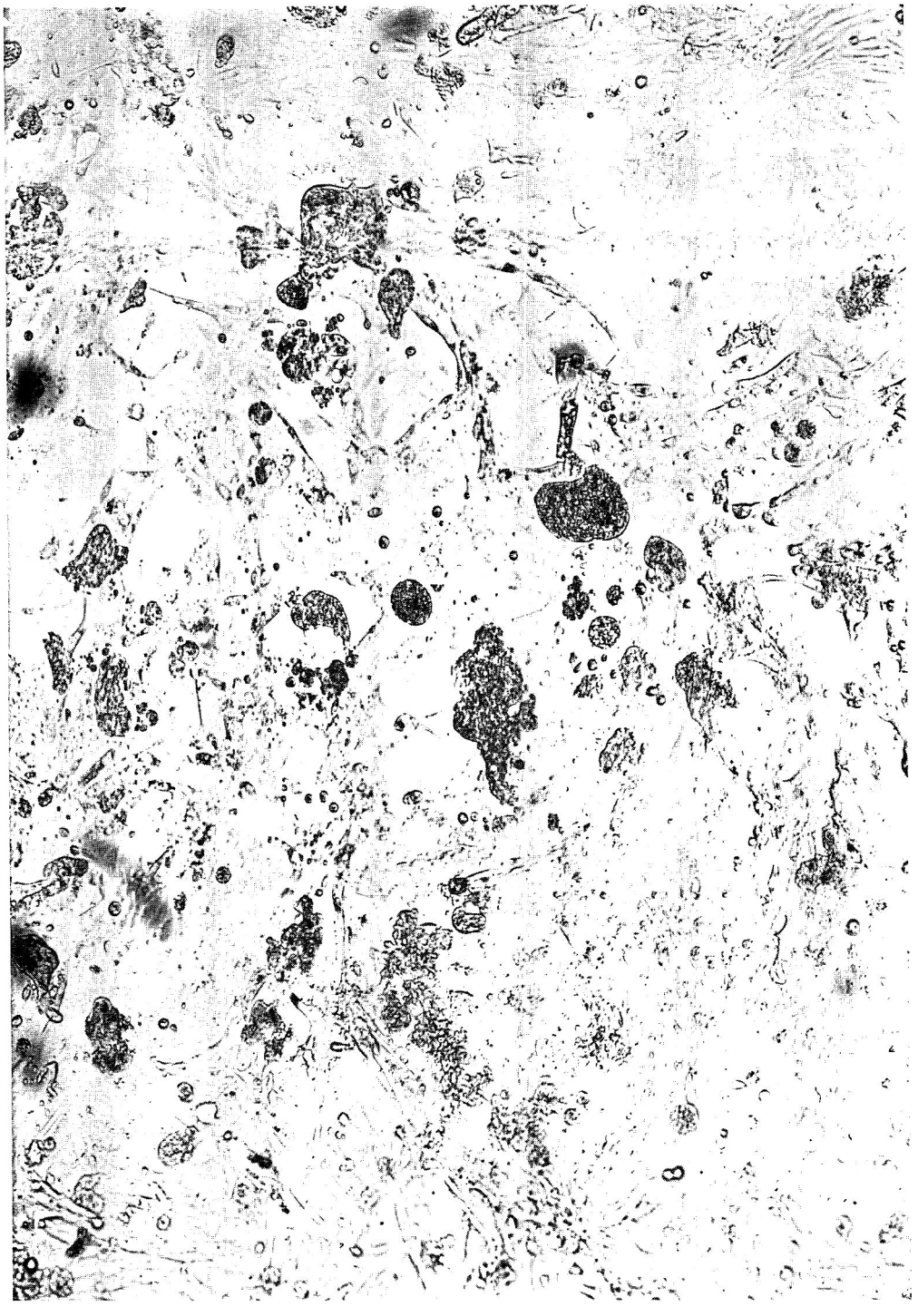


Fig. 2



G. PLUMMER Fig. 3

G. PLUMMER Fig. 4

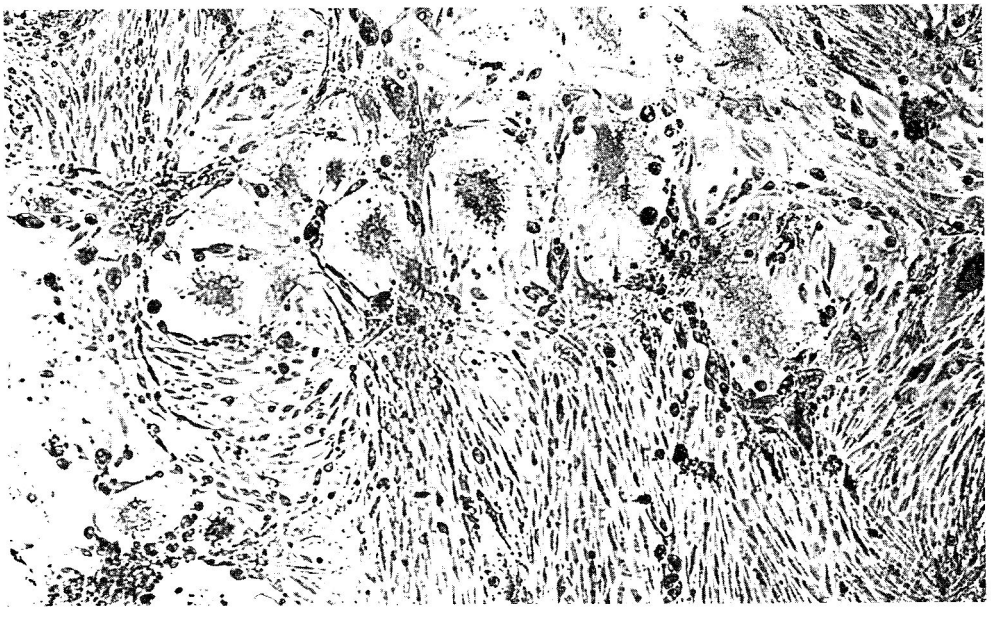
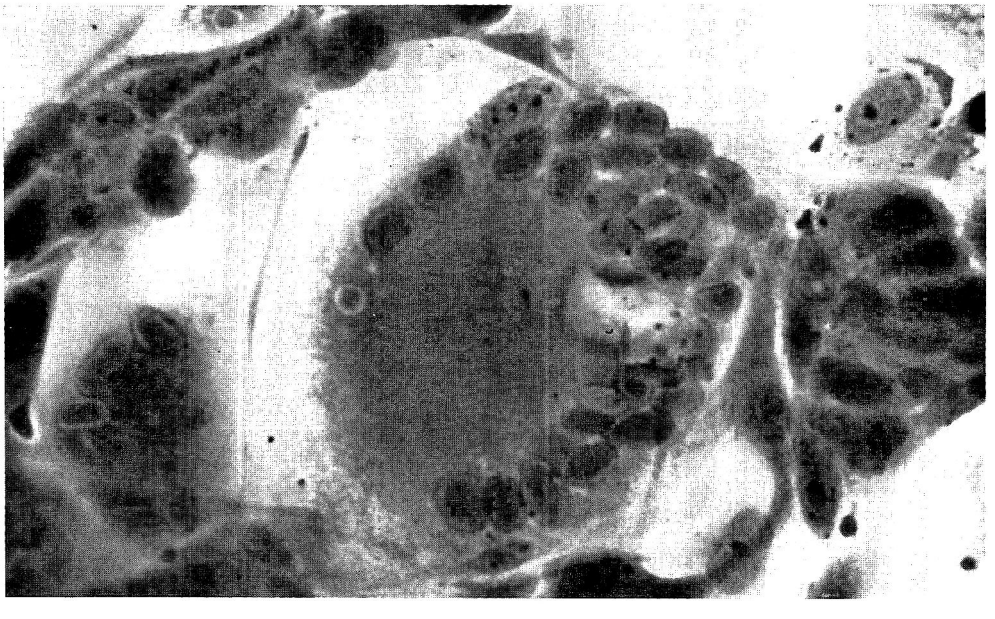


Fig. 5



G. PLUMMER Fig. 6

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Foamy lesions in an unstained rhesus kidney culture. × 46.

Fig. 2. Foamy lesion stained with haematoxylin eosin following fixation with Bouin's fluid. × 330.

PLATE 2

Fig. 3. SV5 lesions in an unstained rhesus kidney culture. × 160.

PLATE 3

Fig. 4. Foamy lesion in an unstained rhesus kidney culture. × 120.

PLATE 4

Fig. 5. MINIA lesions in an unstained rhesus kidney culture. × 80.

Fig. 6. MINIA lesion stained with haematoxylin eosin following fixation with Bouin's fluid. × 495.

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With 1 *plate*
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Bacillus larvae: its Cultivation *in vitro* and its Growth *in vivo*

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(*Received* 6 *April* 1962)

SUMMARY

Spores of *Bacillus larvae* White germinate and make initial vegetative growth best in a limited range of low redox potentials, but later growth and sporulation occur best aerobically. Different media needed for best results with each phase of development of the bacillus are described. Spores of *B. larvae* germinate in the mid-gut contents of honey-bee larvae up to 2 days old. The vegetative forms then migrate and become closely applied to, but do not penetrate, the mid-gut epithelium. Most organisms seem to be voided with the contents of the intestine when an infected larva defaecates shortly before it pupates. A few organisms are presumably left in the intestine and probably invade the tissues of the larva as it pupates.

INTRODUCTION

Bacillus larvae White does not germinate or sporulate on ordinary bacteriological media. Extract of larval honey-bees (White, 1907), unheated egg yolk (White, 1920) and egg yolk + yeast + carrot-extract + peptone (Sturtevant, 1932) are usually fairly suitable media but only when inoculated with many million spores (Tarr, 1937). A medium containing glucose, peptone and thiamine with trace elements (Lochhead, 1942) is sometimes satisfactory and becomes more reliable with added soluble starch or after treatment with activated charcoal (Foster, Hardwick & Guirard, 1950). Good sporulation has been obtained on agar containing extract of pollen (Smith, Beck & Anderson, 1949) and on agar containing yeast extract + soluble starch (Foster *et al.* 1950); good germination has been obtained below the surface of semi-solid agar of other media (White, 1920; Lochhead, 1933).

Rods, presumed to be vegetative forms of *Bacillus larvae*, appear in the intestines of 1 to 2-day-old larval honey-bees within 24 hr. of their food being inoculated with spores (Woodrow & Holst, 1942; Kitaoka, Yajima & Azuma, 1959). The rods are thought not to multiply much in the intestine (Maassen, 1908; Holst, 1946) but to invade the haemolymph where they multiply greatly (Jaeckel, 1930). It is not clear, however, when they leave the gut. Rods have been found in the haemolymph of 2- or 3-day larvae (Jaeckel, 1930) but, judging by the accounts, the larvae, which were artificially and probably very heavily infected, seem to have been moribund, perhaps dead, when examined. (Most larvae die from natural infection immediately before or just after they pupate, when they are about 9 days old and have been sealed in their honeycomb cells.) Other workers have found very few rods in larvae between 3 and 6 days after these had been infected when 0–1 day old, and none when the larvae had become 7–9 days old, yet all remaining infected larvae became diseased when 13–14 days old (Kitaoka *et al.* 1959). Larvae older than 2 days are

immune to infection (by 10^5 spores); larvae younger than 24 hr. are most susceptible and ten or fewer spores then suffice to cause disease (Woodrow, 1942; Hitchcock, 1958). Larvae 4–5 days old are usually immune, but inoculation with very many spores (about 10^7) may infect them (Sturtevant, 1932).

The present paper gives results obtained *in vitro* with modifications of a medium based on one which proved successful for *Streptococcus pluton* (Bailey, 1957; Bailey & Gibbs, 1962) a fastidious pathogen that grows in the same natural environment (intestinal contents of honey-bee larvae) as that in which *Bacillus larvae* starts its life cycle. Observations are given, in the light of these results, on the natural growth of *B. larvae*.

METHODS

Spores of *Bacillus larvae* were obtained from the dried remains (scales) of larvae which had died of American foulbrood. There were about $2{\cdot}5 \times 10^9$ spores/scale but usually no other micro-organisms. Scales were picked out of honeycombs of diseased brood and suitable inocula prepared by extracting them with sterile water.

A basal medium of 1 % (w/v) yeast extract (Difco) + 1 % (w/v) soluble starch was used and the media to be tested were made up from it as follows:

(1) basal + 0·1 M-KH_2PO_4; (2) basal + 0·1 M-KH_2PO_4 + 1 % (w/v) glucose; (3) basal + 0·1 M-KH_2PO_4 + 1 % glucose autoclaved separately; (4) basal + 0·01 M-KH_2PO_4; (5) basal + 0·01 M-KH_2PO_4 + 1 % (w/v) glucose; (6) basal + 0·01 M-KH_2PO_4 + 1 % (w/v) glucose autoclaved separately.

Media were adjusted to pH 6·6 with KOH and autoclaved in closed screw-capped bottles at 116° for 20 min. Unless stated otherwise, inoculated media were incubated aerobically at 34°.

The stabilities of the redox potentials in sterile deep agars of these media were deduced by observing the reduction of methylene blue (0·2 mg./100 ml. medium) in them while they were incubated for several days.

Numbers of organisms were counted in a Helber counting chamber; the proportions of spores to vegetative forms in cultures were found by differentially staining microscope preparations by the malachite green-mercurochrome method of Wynne (1948).

Suitable larvae for infection were obtained by confining the queen of a colony to a comb in a metal cage with slots through which the worker bees could pass but the queen could not. The cage was placed in the brood-nest of the colony and, after 24 hr., the queen was released. After a further 3 days the comb usually contained many hundreds of larvae 0–24 hr. old. Individual larvae were identified with co-ordinates by using a calibrated frame. The food surrounding identified larvae was inoculated by means of a microsyringe with 0·4 μl. of a suspension of spores of *Bacillus larvae* in a 25 % (v/v) solution of honey in water. Each larva received about 10^6 spores. Larvae to be examined histologically were fixed in Bouin, sectioned and stained with Heidenhain's haematoxylin. Larvae not taken for histological or other work were left in the colony until sealed in their cells. The comb was then removed and incubated at 34° for 7 days, after which all larvae were examined and dead ones examined for spores of *B. larvae*.

RESULTS

Semi-solid agar was best for germination of spores; medium 5 (yeast extract + soluble starch + 0·01 M-KH_2PO_4 + 1 % glucose) was clearly superior to the rest: almost every spore germinated in the zone where growth began (Table 1). When there were very few spores/ml. medium, many satellite colonies formed round those colonies which first appeared. Growth appeared on the surface of semi-solid agar after a further 1 or 2 days, the longest time being taken in agars with fewest spores. Growth below the surface then dwindled in media 2 and 5 and vanished in the others, the agar becoming quite clear again. These tests were repeated on many occasions and gave entirely consistent results.

Table 1. *Germination of spores of* Bacillus larvae *in semi-solid agar*

Medium no.	Incubation period (days)	Size of inoculum (approx. log. spores/ml. medium) 8	7	6	5	4	3	2	1	0	Remarks
1	2	+	−	−	−	−	−	−	−	−	Growth began at various depths
	3	+	−	−	−	−	−	−	−	−	
2	2	+	−	−	−	−	−	−	−	−	Growth began between 3 and 5 mm. below the surface and was typically in two very narrow bands
	3	+	+	+	+	+	+	−	−	−	
3	2	+	−	−	−	−	−	−	−	−	As for medium 1
	3	+	−	−	−	−	−	−	−	−	
4	2	+	+	−	−	−	−	−	−	−	As for medium 1
	3	+	+	−	−	−	−	−	−	−	
5	2	+	+	+	+	+	+	−	−	−	Growth began between 5 and 10 mm. below the surface. Two weakly differentiated bands of optimum growth sometimes seen
	3	+	+	+	+	+	+	+	+	+	
6	2	+	+	−	−	−	−	−	−	−	As for medium 1
	3	+	+	−	−	−	−	−	−	−	

Germination was fairly good in deep solid agar, particularly with concentrated inocula. In medium 2 germination and initial vegetative growth were restricted to a narrow zone that was typically in two very narrow horizontal bands. In media 3, 4, 5 and 6 growth often extended from the surface to the bottom of the tube with a wide zone free from growth between 1 and 2 cm. below the surface. The vegetative growth, which appeared after about 4 days, disappeared after another day or two except on or near the surface.

Spores usually failed to germinate in streaks or pour-plates. Most success was with media 3, 5 or 6. When growth appeared in pour-plates it was at first very patchy and few colonies grew on the surface. Inocula of many million spores were necessary to start growth.

Attempts to subcultivate from deep solid agar to the surface of agar of any kind almost always failed, but vegetative growth was readily transferred from the surface or subsurface of semi-solid agar to the surface of agars of most of the media

(Table 2). Vegetative growth could be transferred readily from semi-solid agar for at least 8 days after it had appeared.

Sporulation occurred on the surface of agar or in semi-solid agar of all media except no. 2 or nutrient agar (Table 2), and was best on media 1 or 4, on which sporulation ceased about 10 days after growth began.

Slopes of 10 ml. of medium 4 yielded $2{\cdot}7 \times 10^8$ spores (20 observations; standard error of mean = $0{\cdot}4 \times 10^8$). The average ratio of spores to vegetative forms which developed ultimately in medium 4 was about 1:1. This ratio was increased significantly ($P < 0{\cdot}02$) to about 2:1 when the medium contained 10 % (v/v) of an aqueous extract of pollen (10 %, w/w), freshly collected by bees.

Table 2. *Growth and sporulation of* Bacillus larvae *on agar*

	Growth (1–2 days)		Sporulation	
Medium no.	Transferred from semi-solid agar	Transferred from surface growth	After 4 days	After 6 days
1	+	+	++	++
2	−	++	−	−
3	++	++	−	±
4	+	+	++	++
5	±	++	−	+
6	++	++	−	±
Nutrient agar (Oxoid No. 2)	±	±	−	−

− = growth/sporulation never occurred; ± = growth/sporulation varied between nil and some; + = growth/sporulation always occurred; ++ = growth/sporulation always plentiful.

Table 3. *The numbers of honey-bee larvae which suffered various fates after the food of each had been inoculated with* 10^6 *spores of* Bacillus larvae

	Age (days) of larvae when their food was inoculated			
Fate of larvae	0–1	1–2	2–3	3–4
Pupated normally	3	15	36	52
Ejected before being sealed in their cells	101	39	14	26
Died after being sealed in their cells	11 (11)*	7 (7)*	0	0
Taken when 2–3 days old and examined histologically	5 (5)†	.	.	.
Taken when 4–5 days old and examined histologically	16 (13)†	13 (6)†	10 (0)†	10 (0)†
Taken when 7–8 days old and examined histologically	.	10 (0)†	.	.

* Number in parentheses = larvae with spores of *B. larvae* in body tissues.

† Number in parentheses = larvae containing vegetative forms of *B. larvae* (when present vegetative forms were in the gut contents only).

Results identical with all those above were obtained with added CO_2 (about 5 or 10 %, v/v) in the atmosphere and with media that contained sodium phosphate instead of potassium phosphate. Growth did not occur anaerobically (in McIntosh and Fildes jars), with or without CO_2.

Methylene blue was abruptly reduced below a boundary that stayed about 8 mm. deep for many days in medium 2 agar. It was less abruptly reduced below a boundary that stayed about 20 mm. deep in medium 5 agar, rather gradually reduced below unstable boundaries about 25–35 mm. deep in agars of media 3 and 6, and was not reduced, or only temporarily so below about 35–45 mm., in agars of media 1 and 4.

Spores germinated and produced detectable vegetative growth only in larvae younger than 2 days (Table 3). Histological sections showed that vegetative rods were dispersed evenly throughout the intestinal contents of these young infected larvae. By the time larvae were 4–5 days old, the rods were mostly in a thin layer against the epithelium (Pl. 1, fig. 1). Rods were frequently seen interspaced among and aligned with the hairs of the brush border of the epithelial cells (Pl. 1, fig. 2), but no rod was ever seen to have penetrated the tissue. There is little doubt, however, that many or most of these larvae would have been killed by the infection had they been left to pupate (Table 3).

Some larvae which had been infected between the ages of 1 and 2 days were removed from the comb when 5 days old and incubated in dishes at 34°. They spun cocoons and voided their intestinal contents normally, and their faeces contained very many rods, apparently of *Bacillus larvae*. Most of these larvae then died and spores formed in them.

DISCUSSION

The banded growth of *Bacillus larvae* in deep agar resembled that of *Streptococcus pluton* in the same media (Bailey & Gibbs, 1962) but was slightly nearer the surface. The redox potentials in medium 2 agar, in which the very narrow bands of growth formed, were evidently very stable and it seems that a narrow range of potential, slightly above zero, is required for spores of *B. larvae* to germinate. Medium 5 agar is most suitable for germination of spores of *B. larvae* perhaps because it has a fairly stable wide range of suitable redox potential. When there was double-banded growth, the medium close to the band that first appeared may have become too reduced for the spores in it to germinate. The necessary redox potential presumably occurred again where this reduction was balanced by oxidation from above so that further spores germinated.

Growth soon became limited to the surface or subsurface regions of deep agar of all media, and particularly of those with weakly poised Eh values, probably because the deeper regions became too reduced by the vegetative growth. Organisms migrated through semi-solid agar, hence the satellite colonies and, perhaps, the eventual surface growth on semi-solid agar that had been inoculated with very few spores; but the disappearance of vegetative growth from the deepest regions may have been mostly by autolysis.

Vegetative growth transferred poorly from semi-solid agar to the surface of agars of media 2 and 5, particularly medium 2, probably because the surface of these media becomes very oxidized. (There is evidence that this sometimes happens with medium 2 to make it unsatisfactory for *Streptococcus pluton*; see Bailey & Gibbs, 1962). Vegetative forms of *Bacillus larvae* grew well when transferred to the surface of the other media, probably because they could reduce the weakly poised redox potential of the agar sufficiently to continue their growth. Once vigorous growth was established subcultivation to the surface of any of the media became possible.

The results obtained by previous workers with a range of complex media for *B. larvae* probably depended largely on the redox potentials in the media rather than on their nutritive value and ionic composition.

Adding pollen extract to medium 4 increased sporulation much less than observed by Smith *et al.* (1949), who used a similar medium + Neopeptone (Difco), so that it can be inferred that pollen extract not only improves sporulation but counteracts a detrimental effect of Neopeptone.

The behaviour of *Bacillus larvae* in nature may be interpreted in terms of its characteristics *in vitro*. Spores germinate and multiplication begins *in vitro* within a narrow range of redox potentials. Such conditions may occur only in the young larvae and account for their susceptibility. Old larvae, because of their greatly increased size, may have a lower Eh value in their gut contents, or it may decrease quicker when vegetative *B. larvae* is present, than in young larvae: this could account for the failure either of spores to germinate or of the aerophilic vegetative forms to migrate to the epithelium in old larvae. (It would also account for the ability of *Streptococcus pluton*, which needs a microaerobic to anaerobic environment, to continue its growth in the gut contents of larvae of any age; see Bailey & Gibbs, 1962.) The vegetative forms of *B. larvae* that reached the gut epithelium were unable to penetrate the tissues, at least of the larvae that were not ejected by nurse bees, and most of them were probably voided in the faeces when the larvae pupated. These vegetative forms are of no further consequence because they cannot infect more larvae (Tarr, 1937) even if they could survive desiccation in the larval faeces. A few organisms presumably remain in the gut and invade the aerobic tissues where they can multiply and sporulate, probably when the gut epithelium changes to the adult type during pupation.

Bacillus larvae may have penetrated the tissues of the many young larvae that were ejected shortly after they had been infected (Table 3). If this always happened, however, *B. larvae* could not survive because the larvae were ejected before they had begun to decompose and spores would not have formed.

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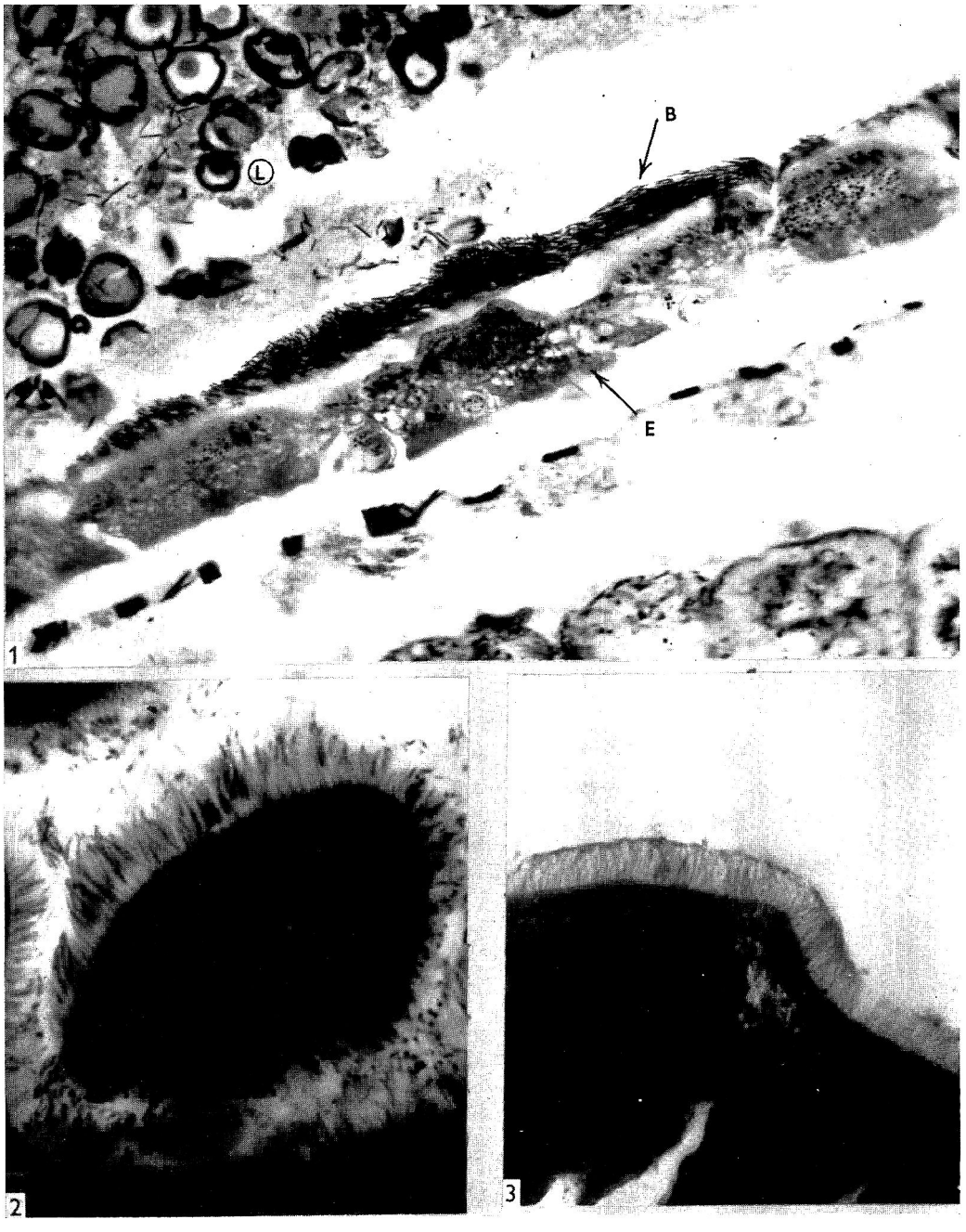
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EXPLANATION OF PLATE

Fig. 1. *Bacillus larvae* in the mid-gut of a 4- to 5-day honeybee larva. (×400.) L = lumen of gut, B = rods of *Bacillus larvae*; E = gut epithelium.

Fig. 2. *Bacillus larvae* among the hairs of the brush border of an epithelial cell. (× 1000).

Fig. 3. Brush border of a normal epithelial cell.

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Effect of Chilling on *Aerobacter aerogenes* in Aqueous Suspension

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SUMMARY

The lethal effect of cold shock on *Aerobacter aerogenes* suspensions depended on the time of exposure to low temperature, the growth phase, the concentration of bacteria, the diluent. No death occurred when weak suspensions of susceptible bacteria (about 10^8/ml.) in buffered saline (pH 6·5) were rapidly cooled to 0° and immediately warmed to 20°, but loss of viability was progressive during 1 hr. at 0°. Bacteria harvested from defined medium at intervals during the exponential growth phase varied in sensitivity to chilling but were more susceptible than stationary phase organisms. While growing in partially synchronized culture the sensitivity of bacteria did not increase significantly during the division lag phase. The viability of dense suspensions (about 10^{10} bacteria/ml.) in buffered saline was little affected by chilling for 1 hr. at 0°, irrespective of the growth phase. A bacteria-free filtrate from a chilled concentrated suspension of exponential-phase organisms substantially protected a dilute suspension from the lethal effect of chilling. Substances found in protective filtrates were amino acids, adenosine triphosphate and nucleic acid constituents. When added to the diluent in which susceptible bacteria were chilled, a mixture of amino acids afforded some protection; small amounts of adenosine triphosphate had no effect. Other substances found to protect susceptible bacteria were sucrose (0·3M), magnesium or calcium ions (5×10^{-3}M) and, to a much smaller extent, spermine (10^{-5}M). The present results support the suggestion that the lethal effect of chilling is at least partly due to interference with the functioning of a bacterial permeability control mechanism.

INTRODUCTION

Sudden chilling causes loss of viability of suspensions of exponential phase *Escherichia coli* (Sherman & Albus, 1923; Sherman & Cameron, 1934; Hegarty & Weeks, 1940; Meynell, 1958) and *Pseudomonas pyocyanea* (Gorrill & McNeil, 1960). Meynell (1958) showed that the lethal effect was not due to sudden cooling in itself by demonstrating that survival was complete either after gradual cooling in a potentially lethal diluent or after sudden chilling in a solution of sucrose (0·3M). He suggested the lethal effect may be due to interference with an adaptive mechanism which prevents entry of water into the organism and that the mechanism has negligible activity at 4°. Bacterial death following chilling was not accompanied by lysis and Meynell detected no differences between electron micrographs of chilled and unchilled bacteria from the same culture. Gorrill & McNeil (1960) could not distinguish morphological differences between cold shocked and unshocked *P. pyo-*

cyanea with either dark-ground, phase-contrast or ordinary light microscopy. The present paper records a study of cold shock in *Aerobacter aerogenes*.

METHODS

Aerobacter aerogenes strain NCTC 418 was obtained from Professor Sir Cyril Hinshelwood's laboratory.

Medium and cultural conditions. Organisms were grown at 37° and at a continuously maintained pH value of 7·2–7·4 with adequate aeration in a batch culture vessel containing the defined carbon-limiting medium previously described (Strange, Dark & Ness, 1961). Partially synchronized cultures were obtained by depriving organisms of an energy source for a period before growth (McNair Scott & Chu, 1958): medium was seeded with a suspension of washed stationary-phase bacteria which had been held in buffered saline (pH 6·5) for 20 hr. at 37° with aeration.

Viability determinations. The direct determination of the percentage viable bacteria in a suspension by a slide culture method (Postgate, Crumpton & Hunter, 1961) and counts of viable bacteria, were made as previously described (Strange *et al.* 1961).

Chilling. Bacteria were usually separated from the culture by centrifugation and resuspended at a suitable concentration in the same diluent as that in which they were subsequently chilled. With experiments shown in Figs. 1 and 3, culture directly from the growth vessel was used. Rapid cooling of bacterial suspensions was achieved by dilution (1/50–1/100) in cold diluent held in a temperature-controlled bath containing aqueous ethylene glycol at 0°. Concentrated bacterial suspensions were initially cooled to near 0° by contact with brine at −10° before placing in the bath. When freezing occurred during cooling, the suspension was discarded. The diluents used were buffered saline which contained: NaCl (0·13 M) and appropriate concentrations of $K_2HPO_4 + KH_2PO_4$ (0·02 M PO_4) to give the required pH value; 0·05–0·15 M 2-amino-2(hydroxymethyl)-1:3-propanediol(tris) + HCl to give the required pH value (tris buffer); distilled water. All diluents and diluents + additives were filtered through a well-washed filter membrane before use.

Materials. Distilled water was passed through a mixed-bed ion-exchange resin (Amberlite MB-1 from British Drug Houses Ltd.) column before use. Whenever possible, Analytical Reagent Grade substances were used. Hydrated disodium adenosine-5-triphosphate (ATP) was obtained from Sigma Chemical Co., St Louis, Missouri, U.S.A.; dehydrated firefly tails were obtained from L. Light & Co. Ltd.; Oxoid filter membranes, grade A.P., from Oxo Ltd.

Analytical Methods. The total amino acid content of samples was determined by a ninhydrin colorimetric method (Yemm & Cocking, 1955) with alanine as the standard. Amino acids were identified by 2-dimensional paper chromatography with phenol saturated with water in an atmosphere of NH_3 as first solvent, butanol + acetic acid + water (40 + 10 + 50, by vol.; upper phase) as second solvent and ninhydrin as the spray reagent. ATP was determined by the following firefly luminescence technique which is essentially that described by Strehler & Totter (1952): reaction mixtures (4 ml.) contained 0·02 M sodium arsenate buffer (pH 7·4), 3 mM-Mg^{2+}, 0–0·2 μg. ATP and enzyme extracted from 2·5 mg. firefly tails; at the addition of enzyme, a stop-watch was started and luminescence was measured for 0·5 min. (beginning 1 min. after mixing) with a scintillation counter constructed by

our colleague Mr S. Lovett. During a series of ATP determinations, counts were obtained for appropriate blank and standard mixtures at regular intervals. Magnesium was determined by the Eriochrome Black T colorimetric procedure (Levine & Cummings, 1956) and calcium was detected by precipitation as the oxalate. Ultraviolet (u.v.) absorption was measured in a Unicam quartz spectrophotometer, model S.P. 500, with a 1 cm. light path. Bacterial dry weights were determined as previously described (Strange *et al.* 1961).

RESULTS

Influence of bacterial growth phase on the lethal effect of chilling

Figure 1 shows a typical growth curve for *Aerobacter aerogenes* in defined medium and the sensitivity to chilling of bacteria harvested at intervals during the growth period. Exponential-phase organisms were most sensitive to chilling after growth for about 132 min. and then became progressively less sensitive up to 168 min. before division ceased. Differences in the sensitivity of *Escherichia coli* to chilling during exponential growth were reported by Hegarty & Weeks (1940) and Meynell (1958; Fig. 1). Loss of viability of susceptible bacterial suspensions held at 0° was progressive and bacteria removed immediately after chilling were completely viable. Figure 2 shows survival curves for bacteria harvested at four different times during the growth period and then held at 0°. The susceptibility to chilling of bacteria in different stages of the division cycle was examined by means of a partially synchronized culture (Fig. 3). During the initial lag phase, the bacteria became almost completely resistant to chilling but sensitivity increased immediately division began. In this and other experiments resistance to chilling during the first division lag period (i.e. when organisms were increasing in size before the second division) did not increase to that of stationary phase or initial lag-phase bacteria, but remained unchanged or increased only slightly before decreasing during the second division (Fig. 3). It is of interest that, whereas freshly harvested stationary-phase organisms were relatively resistant to chilling, after storage for 20 hr. at 37° in buffered saline with aeration their resistance decreased and was little higher than that of exponential-phase bacteria (Fig. 3, $t = 0$).

Effect of the diluent

In the experiments above the ionic strength of buffered saline used as a diluent was similar to that of 'physiological saline' (NaCl, 0·9%, w/v). When similar concentrations of the same batch of exponential-phase organisms were chilled in this and other diluents, the losses of viability shown in Table 1 were obtained. In several experiments, losses were higher in tris buffer than in the other diluents. The high loss of viability occurring in this diluent during chilling was not due to contamination of tris salt by heavy metals since the addition of disodium ethylenediamine tetraacetic acid (EDTA; 0·32 mM) to tris buffer had no effect on the results. Of these diluents, distilled water was usually the least toxic but with a few batches of organisms a slightly greater loss of viability occurred on chilling in water than in buffered saline.

Loss of viability of bacterial suspensions held at 0° occurred progressively with time (Fig. 2) and it was of interest to determine whether sudden warming after a

period at 0° decreased the rate of viability loss. A suspension of washed exponential-phase organisms in buffered saline (pH 6·5) was cooled to 0° and samples, removed immediately and at intervals, were rapidly warmed to 18° in a water bath. Chilling

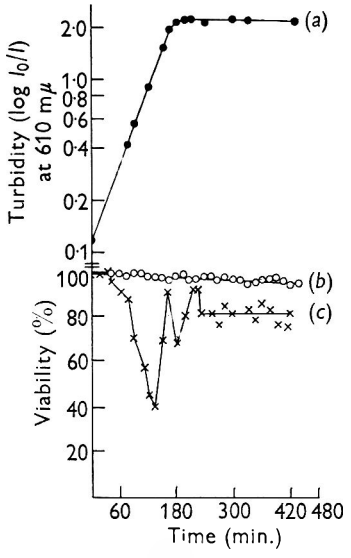

Fig. 1

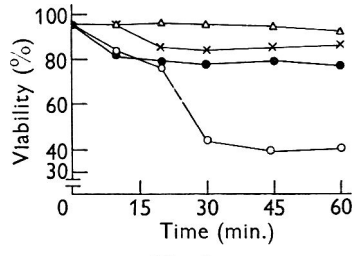

Fig. 2

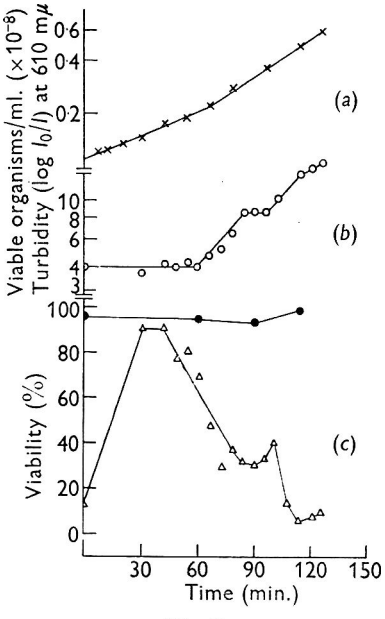

Fig. 3

Fig. 1. Effect of growth phase on susceptibility of *Aerobacter aerogenes* to cold shock. (*a*) Growth curve in carbon-limiting mannitol + salts medium. (*b*) Viabilities of samples 30 min. after dilution to about 10^8 bacteria/ml. in buffered saline held at 20°. (*c*) Viabilities after a similar period at 0°.

Fig. 2. Survival of exponential and stationary phase *Aerobacter aerogenes* at 0°. Bacteria were separated from the culture by centrifugation and diluted to about 10^8/ml. in buffered saline at 0°. Exponential phase organisms (○); bacteria in the stationary phase for 15 min. (●), 75 min. (×), and 135 min. (△).

Fig. 3. Susceptibility of *Aerobacter aerogenes* to cold shock during partially synchronous growth. (*a*) Growth curve in defined carbon-limiting medium determined by viable counts (○) and turbidity (×). (*b*) Viabilities of samples 30 min. after dilution to about 10^8 bacteria/ml. in buffered saline at 20°. (*c*) Viabilities after a similar period at 0°.

followed immediately by warming had no effect on viability, whereas after chilling for 5 min. or more, suspensions continued to lose viability at 18° but at a slower rate than at 0° (Fig. 4).

Influence of bacterial concentration

Five suspensions containing different concentrations of a batch of exponential-phase organisms in buffered saline were held at 0° for 1 hr. Survival curves showed that the death rate was greater the sparser the population (Fig. 5). A similar but less pronounced phenomenon occurred in tris buffer: suspensions of 5×10^7, 10^9 and 10^{10} viable bacteria/ml. tris buffer had viabilities of 8, 57 and 96% respectively, immediately after chilling, and 8, 5 and 53% respectively, after 1 hr. at 0°.

In the case of concentrated exponential-phase suspensions, it seemed possible that the relatively great resistance to chilling was due to the appearance in the suspending fluid of material with protective activity. To examine this possibility,

Table 1. *Effect of the diluent on the loss of viability of exponential phase* Aerobacter aerogenes *held at* 0° *and* 20°

Bacteria separated from the culture by centrifugation were diluted to about 10^8/ml. in diluents held at 0° and 20°.

Experiment no.	Diluent	% viability of suspension after 45 min. at 0°	% viability of suspension after 45 min. at 20°
1	Buffered saline, pH 6·5	32	98
	0·05 M-tris buffer, pH 7·5	1	98
	Distilled water	75	98
2	Buffered saline, pH 6·0	54	99
	Buffered saline, pH 6·5	37	
	Buffered saline, pH 7·0	42	
	Buffered saline, pH 7·5	21	
	0·05 M-tris buffer, pH 7·5	1	99
	0·05 M-tris buffer, pH 7·5 + 0·32 M-EDTA	1	
	Distilled water	56	
3	Buffered saline, pH 6·5	50	99
	0·05 M-tris buffer, pH 7·5	28	98
	0·10 M-tris buffer, pH 7·5	15	97
	0·15 M-tris buffer, pH 7·5	25	97
	Distilled water	85	86
4	Buffered saline, pH 6·5	8	98
	0·05 M-tris buffer, pH 7·5	< 1	99
	Distilled water	94	96

supernatant liquid separated from a chilled (1 hr., 0°) suspension initially containing $1{\cdot}3 \times 10^{10}$ viable exponential-phase bacteria/ml. buffered saline was passed through a filter membrane and tested as follows: tubes containing (*a*) filtrate, (*b*) filtrate + 3 vol. buffered saline, (*c*) buffered saline, were cooled and sufficient fresh suspension was added to give about 10^8 organisms/ml. each diluent. After 45 min. at 0°, the viabilities of (*a*), (*b*) and (*c*) were 99, 94 and 23%, respectively. When filtrates from chilled (1 hr., 0°) exponential- and stationary-phase bacterial suspensions of about the same concentration (10^{10} bacteria/ml.) were tested in a similar manner, viabilities after 45 min. at 0° were 98 and 64%, respectively, as compared with 24% in the buffered saline control. The activity of protective material present in filtrates was not affected by heating for 15 min. at 100°. These and other experiments showed that filtrate from chilled exponential-phase bacteria and, to a lesser extent,

from chilled stationary-phase organisms, contained substances which protected exponential-phase *Aerobacter aerogenes* from the lethal effect of chilling.

Examination of leakage products from chilled bacteria

The identification of the protective material present in filtrates from chilled bacterial suspensions was complicated by the fact that the time taken to prepare the relatively concentrated suspensions required for analytical investigation was

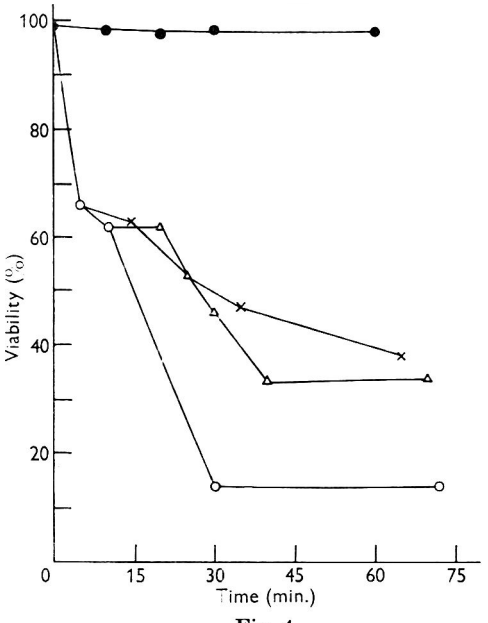

Fig. 4

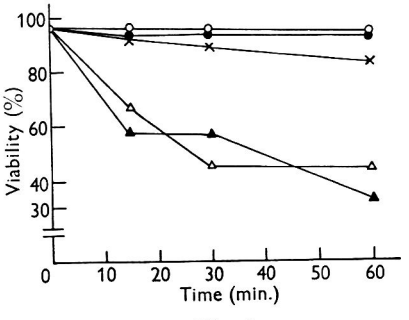

Fig. 5

Fig. 4. Survival of exponential phase *Aerobacter aerogenes* chilled at 0° for short periods and then held at 18°. Bacterial suspensions were prepared as in Fig. 2. Viability at 18° immediately after chilling (●), after chilling for 5 min. (×) and after chilling for 10 min. (△); viability of control suspension held throughout at 0° (○).

Fig. 5. Effect of population density on the survival of exponential phase *Aerobacter aerogenes* at 0°. Bacteria, separated from the culture by centrifugation, were resuspended at different concentrations in buffered saline held at 0°. Viabilities immediately and at intervals after chilling of suspensions initially containing: 6×10^7 (▲), $1{\cdot}2 \times 10^8$ (△), $1{\cdot}2 \times 10^9$ (×), $2{\cdot}4 \times 10^9$ (●), $1{\cdot}2 \times 10^{10}$ (○) viable bacteria/ml.

sufficient to affect the sensitivity of bacteria to chilling. For example, a suspension diluted and chilled 10 min. after the bacteria were harvested lost 60% viability in 45 min. at 0° as compared with 10% when the suspension was held for 45 min. at 20° before chilling for the same period. The problem could not be resolved by direct chilling of the culture because medium constituents and growth products interfered with subsequent analysis. In the experiments described, suspensions were chilled 15–20 min. after harvesting the organisms. Bacteria, separated from an exponential-phase culture by filtration through filter membrane, were washed several times on the pad with buffered saline and resuspended at a concentration of $3{\cdot}2 \times 10^9$ viable bacteria/ml. (99% viable). Part of the suspension was rapidly cooled to 0° and the

remainder held at 20°. Samples were taken immediately and at intervals from each suspension for viability determinations and analyses. The latter samples were immediately freed from bacteria by filtration (filter membrane) and the filtrates analysed for ninhydrin-reacting substances and ATP. The concentration of ninhydrin-reacting substances in filtrates was expressed as percentage total amino acids (as alanine) extractable with cold 0·5 N-$HClO_4$ from an equal volume of whole bacterial suspension. Cooled suspension (4 ml.) was acidified by the addition of $HClO_4$ (72 %; 0·17 ml.) and left for 30 min. at 0°. After centrifugation, the supernatant fluid was neutralized with 2 N-KOH, filtered through a membrane filter and analysed for amino acids. Total amino acids (as alanine) in exponential phase *Aerobacter aerogenes* suspensions accounted for about 1 % of the bacterial dry weight. The results (Fig. 6) showed that the chilled suspension progressively lost viability, whereas the unchilled suspension remained completely viable, and the leakage of ninhydrin-reacting substances and also of ATP was greater from chilled bacteria. Paper chromatographic examination of similar filtrates obtained from more concentrated bacterial suspensions showed that the ninhydrin-reacting material could be largely accounted for as free amino acids and peptides of relatively small molecular weight. After electrolytic desalting and concentration, filtrate from a chilled suspension (1 hr., 0°) gave spots corresponding in position to aspartic acid, glutamic acid, glycine, serine, alanine, methionine, valine, leucine, arginine and histidine, with at least two unidentified components; acid hydrolysis of the filtrate (6 N-HCl, 20 hr., 106°) caused an increase in intensity of several spots, the disappearance of two unidentified constituents and the appearance of several other common amino acid spots. Paper chromatograms of filtrate from suspensions of similar concentration held for the same period at 20° showed the presence of several amino acids but in much lower concentrations than those found with chilled suspensions. Neither acid-insoluble protein nor ribonucleic acid (RNA) were detected as constituents of the material which leaked from chilled bacteria. For example, filtrate from a chilled exponential-phase suspension of organisms equivalent to 26 mg. dry weight/ml. (about 5×10^{10} bacteria/ml.) gave no turbidity with $HClO_4$ (0·5 N) under conditions where protein or RNA equivalent to 0·04 % bacterial dry weight would have been detected. Although acid-insoluble RNA was absent, filtrates from chilled and unchilled bacterial suspensions contained u.v.-absorbing substances in greater concentration than could be accounted for by ATP. Maximum absorption occurred at a wavelength near 260 mμ and the initial rate of leakage of these substances was much greater from chilled than from unchilled organisms (Fig. 7). However, on storage of suspensions for longer periods at 20° the u.v.-absorption of the suspending liquid increased steadily, whereas at 0° it reached a maximum after about 30 min. and then remained unchanged. This was due to metabolism of endogenous RNA which occurs in starved *Aerobacter aerogenes* suspensions held at 20°, resulting in the excretion of nucleic acid bases (Strange *et al.* 1961), whereas at 0°, negligible degradation of RNA was found to occur. Neither magnesium nor calcium ions were found in protective filtrates under conditions where 0·5 mM of either would have been detected.

Protective effect of exogenous substances

The possibility that the protective effect of leakage products from chilled organisms was due to the presence of ATP or amino acids was investigated. Also, the influence during cold shock of various other substances, not detected in leakage products, was examined.

ATP. The addition of ATP at a concentration similar to that found in protective filtrates (10–20 μM) to buffered saline or tris buffer diluents afforded no protection to exponential phase organisms against cold shock.

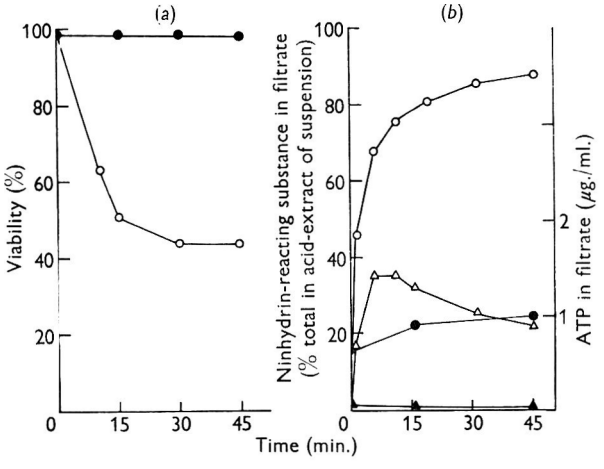

Fig. 6

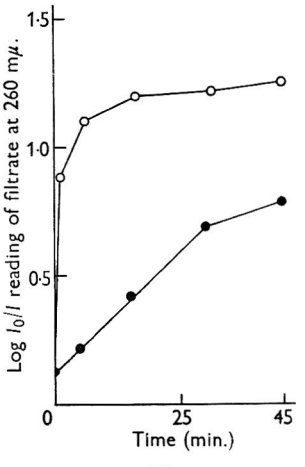

Fig. 7

Fig. 6. Leakage of endogenous constituents from exponential phase *Aerobacter aerogenes* held in suspension at 0° and 20°. Washed organisms were resuspended in buffered saline (equiv. 2·1 mg. bacterial dry weight/ml.). (*a*) Loss of viability at 0° (○) and 20° (●). (*b*) Concentration of ninhydrin-reacting substances as alanine (percentage total cold acid-extractable amino acids in whole suspension) in filtrate from suspension at 0° (○) and 20° (●); ATP (μg./ml.) in filtrate from suspension at 0° (△) and at 20° (▲).

Fig. 7. Leakage of u.v.-absorbing substances from exponential *Aerobacter aerogenes* held in suspension at 0° and 20°. Bacterial suspension (equiv. 2·6 mg. bacterial dry weight/ml.) was prepared as in Fig. 6. Spectrophotometric measurements at 260 mμ on filtrate from suspension at 0° (○) and 20° (●).

Amino acids. Loss of viability of bacteria chilled in buffered saline containing the 10 amino acids detected in leakage products (each 0·5 mM) occurred at a slower rate than in buffered saline alone (Fig. 8). The addition of 7 other amino acids (each 0·5 mM) to this mixture did not improve the protection afforded and neither mixture was as effective as leakage products (Fig. 8). The influence of the 17 amino acid mixture was similar after fivefold dilution with buffered saline, but below this concentration the protective effect disappeared.

Divalent metal ions. The leakage of endogenous constituents which occurred as a result of cold shock indicated that a permeability control mechanism was affected by chilling; such a mechanism would presumably be located in the cytoplasmic membrane. The known stabilizing effect of magnesium ions on isolated protoplast membranes (Weibull, 1956) and spheroplasts (Lederberg, 1956; McQuillen, 1958)

suggested that metal ions might protect bacteria during cold shock. The effect of adding magnesium and other divalent metals to the diluent in which bacteria were chilled was investigated. Bacteria suspended in buffered saline or tris buffer were substantially protected during chilling in the presence of Mg^{2+}, Ca^{2+} or Mn^{2+} (Table 2; Fig. 9). With distilled water as the diluent, Mn^{2+} was slightly toxic, Ca^{2+} or Mg^{2+} had little effect and Co^{2+}, Zn^{2+} or Fe^{2+} were very toxic (Table 2). The pH value of suspensions in distilled water was decreased by the presence of metals; this may have contributed to the loss of viability which occurred.

Sucrose and erythritol. Meynell (1958) showed that exponential-phase *Escherichia coli* suspensions were protected during chilling by sucrose (0·3 M). Chilled *Aerobacter*

Table 2. *Effect of divalent metal ions on the loss of viability of exponential-phase* Aerobacter aerogenes *suspensions at* 0°

Bacteria, separated from the culture by centrifugation, were diluted to about 10^8/ml. in various diluents.

Bacteria batch no.	Diluent	Addition to diluent	% viability of suspension after 45 min. at 0°
1	buffered saline (pH 6·5)	nil	10
		$MgSO_4$: 0·5, 1·0, 5·0 mM	19, 16, 53
		*$MnSO_4$: 0·5, 1·0, 5·0 mM	7, 8, 84
		$CaCl_2$: 0·5, 1·0, 5·0 mM	10, 16, 71
2	0·05 M-tris buffer (pH 7·5)	nil	< 1
		$MgSO_4$, 5 mM	94
		$MnSO_4$, 5 mM	74
		$CaCl_2$, 5 mM	88
3	distilled water	nil	85
		$MgSO_4$: 1·0, 2·5, 5·0 mM	76, 79, 83
		$MnSO_4$: 1·0, 2·5, 5·0 mM	28, 37, 57
		$CaCl_2$: 1·0, 2·5, 5·0 mM	88, 81, 91
		$CoSO_4$: 1·0, 2·5, 5·0 mM	< 1
		$ZnSO_4$: 1·0, 2·5, 5·0 mM	< 1
		$FeSO_4$: 1·0, 2·5, 5·0 mM	< 1

* Precipitation occurred when Mn^{2+} was added to this diluent.

aerogenes suspensions were also protected by sucrose (Fig. 10). Analysis of filtrates of chilled bacterial suspensions in buffered saline with and without sucrose (0·3 M) showed that the concentration of leakage products was considerably less in the presence of sucrose. This sugar does not penetrate into *A. aerogenes* whereas L-erythritol does (Postgate & Hunter, 1961). When bacteria were chilled in buffered saline, buffered saline + sucrose (0·3 M) and buffered saline + erythritol (0·3 M), losses of viability after 1 hr. at 0° were 99, 24 and 88 %, respectively.

Spermine. Since spermine (10^{-3} M) stabilizes *Pasteurella tularensis* and *Escherichia coli* spheroplasts against osmotic damage (Mager, 1959) it was possible that this substance might protect bacteria during cold shock. At 0°, the loss of viability of exponential-phase *Aerobacter aerogenes* in buffered saline containing 10^{-3} M spermine was more rapid than in buffered saline alone. In the presence of 10^{-5} M spermine there was a transient protective effect: losses of viability after 10, 20 and 30 min. at

0° were 34, 65 and 75%, respectively, as compared with 78, 82 and 86%, respectively, in the control suspension.

Revival of chilled bacteria

Protective substances which appeared to exert their influence on bacteria during chilling may have in fact acted after chilling, by reviving moribund bacteria so that they were able to grow on nutrient agar. When bacterial suspensions in buffered saline were chilled and then amino acids, magnesium, calcium or manganese added, viability was not affected. However, when leakage products from a chilled concentrated suspension were added to a dilute chilled suspension, the viability increased. A suspension of *Aerobacter aerogenes* in buffered saline (3×10^8 bacteria/ml.) was held at 0° and samples, removed at intervals, were diluted with an equal volume of either buffered saline or filtrate from chilled (1 hr., 0°) exponential-phase bacteria in buffered saline (3×10^{10}/ml.). Viabilities, determined immediately after dilution with buffered saline, of bacteria held at 0° for 15, 30 and 60 min. were 39, 23 and 15%, respectively; after dilution with filtrate, the viabilities of the samples were 76, 72 and 35%, respectively. On chilling similar concentrations of these bacteria in equal volumes of buffered saline and filtrate, viabilities after the same periods at 0° were 98, 92, and 89%, respectively. Thus, the protective effect of leakage products exerted during chilling was greater than the revival effect which

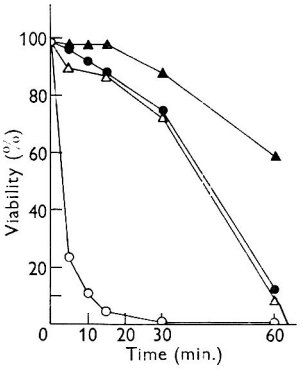

Fig. 8

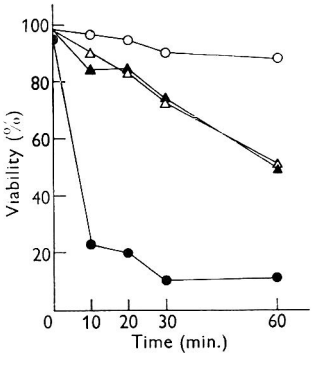

Fig. 9

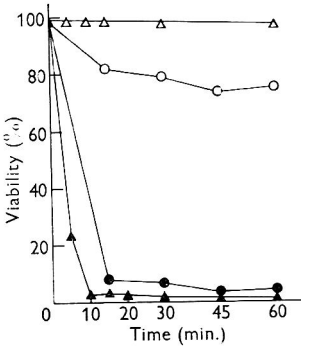

Fig. 10

Fig. 8. Influence of leakage products from chilled bacteria and amino acid mixtures on the survival of exponential phase *Aerobacter aerogenes* at 0°. Bacteria separated from the culture were diluted to about 10^8/ml. in the solutions given held at 0°. Viability in filtrate (▲), in amino acid mixture A (△), in amino acid mixture B (●) and in buffered saline (○). The filtrate was prepared from a suspension of chilled exponential phase organisms in buffered saline ($1{\cdot}3 \times 10^{10}$/ml.). Mixture A contained 0·5mM each of the 10 amino acids (L-isomers) found in leakage products (see text) dissolved in buffered saline: mixture B contained amino acids in A + 0·5mM each of tyrosine, phenylalanine, lysine, α, ε-diaminopimelic acid (meso), threonine (DL), cystine and tryptophan.

Fig. 9. Influence of divalent metal ions on the survival of exponential *Aerobacter aerogenes* at 0°. Viability of suspensions (about 10^8 bacteria/ml.) in tris buffer containing: 5mM-$MgSO_4$(○), 5mM-$CaCl_2$ (△), 5mM-$MnSO_4$ (▲); viability in tris buffer control (●).

Fig. 10. Influence of sucrose on the survival of exponential phase *Aerobacter aerogenes* at 0°. Bacteria separated from two separate cultures were diluted to about 10^8/ml. in diluents at 0°. Viability in buffered saline containing 0·3M sucrose (○, △), in buffered saline control (●, ▲).

these substances exerted on chilled bacteria. The revival effect was not due to transfer of leakage products with bacteria onto slide cultures; in experiments where the viability was determined by viable counts involving a $1/10^5$ dilution of the sample before plating, an increase in the viability of a chilled suspension occurred after the addition of leakage products.

DISCUSSION

The effect of the growth phase on the susceptibility of dilute *Aerobacter aerogenes* suspensions to cold shock was similar to that found with *Escherichia coli* (Sherman & Albus, 1923; Sherman & Cameron, 1934; Hegarty & Weeks, 1940; Meynell, 1958) and *Pseudomonas pyocyanea* (Gorrill & McNeil, 1960). Also, as previously found, the lethal effect of cold shock depended on the diluent. However, with exponential-phase *A. aerogenes* the lethal effect was less in distilled water than in buffered saline or tris buffer, whereas with growing *P. pyocyanea* it was greater in distilled water than in Ringer's solution, buffer solution or saline (Gorrill & McNeil, 1960). In this connexion, it is of interest that the death rate of steadily growing *A. aerogenes* caused by freezing in liquid nitrogen was less with suspensions in distilled water than with those in saline phosphate or saline tris buffers (Postgate & Hunter, 1961).

The finding that a rapid leakage of endogenous constituents occurs on chilling bacteria supports the view that the lethal effect of cold shock is due to interference with a bacterial permeability control mechanism (Meynell, 1958). The population density phenomenon, and the protective effect that filtrates of dense populations exert on the survival of sparser populations, together imply that one or more of these leakage products is necessary for their survival and can be resorbed by the bacteria from their external environment. This resorption must be a rapid process, since brief exposure of moribund populations in buffered saline to leakage products from a denser population increased their viability. This reactivation phenomenon was not only observed when viability was determined by slide culture but also in experiments where viability was determined by a plate counting technique involving a $1/10^5$ dilution of the suspension. The protective material clearly induced a permanent change in the population; one no longer influenced by dilution.

Our experiments show that during chilling the protective activity of leakage products may be partially due to the presence of amino acids; ATP was also leaked but it was inactive when added to diluents in which bacteria were chilled. Other substances which protected bacteria but which were not detected or are unlikely to be constituents of leakage products are calcium, magnesium, manganese and sucrose. The mechanisms by which these substances protect during cold shock are probably different from the one which operates with leakage products or amino acids. The known stabilizing effect of magnesium on isolated protoplast membranes (Weibull, 1956) and spheroplasts (Lederberg, 1956; McQuillen, 1958) suggests that the metal ions decrease the permeability of the cytoplasmic membrane in the intact cell during chilling. The protective influence of sucrose shown previously with *Escherichia coli* by Meynell (1958) is probably due to its osmotic activity which causes a decrease in the rate of diffusion of constituents out of bacteria. Erythritol in equivalent concentration did not have a similar protective effect, presumably because this substance is osmotically neutral with *Aerobacter aerogenes* (Postgate & Hunter, 1961).

Despite its reported stabilizing effect on *Escherichia coli* spheroplasts against osmotic shock (Mager, 1959), low concentrations of spermine had only a transient protective effect on chilled *Aerobacter aerogenes*. However, higher concentrations of spermine were toxic to chilled organisms and the losses of viability which occurred with lower concentrations of the substance may have been due to this toxic effect as well as to cold shock.

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The Serological Grouping of *Streptococcus equinus*

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SUMMARY

Streptococci isolated from horse faeces and corresponding to the description of *Streptococcus equinus* Andrewes & Horder (1906) were found to belong to the serological group D. The group D antigen was produced by all the strains examined, but was not always extractable from whole organisms by HCl or formamide; broken organisms, however, always gave group D antigen. Collections of *S. equinus* and *S. bovis* strains were found to be physiologically similar, but distinguishable.

INTRODUCTION

Andrewes & Horder (1906) first described *Streptococcus equinus* as the predominant streptococcus of horse faeces. A fuller description was given by Hodge & Sherman (1937; see Table 1). The serology of *S. equinus* has received little attention. According to *Bergey's Manual* (1957) 'no group-specific antigen has been demonstrated'. However, Sherman (1938) reported that some of his strains of *S. equinus* 'reacted weakly' with group D antiserum and there have also been recent reports of *S. equinus* strains reacting with group D antisera (e.g. Fuller *et al.* 1960; Raibaud, Caulet, Galpin & Mocquot, 1961). These observations are substantiated by our present findings with 50 strains of *S. equinus* isolated from horse faeces. Because *S. equinus* is similar in many respects to *S. bovis*, which also belongs to serological group D (Shattock, 1949), the physiological reactions of the two species were compared.

METHODS

Organisms. Thirty-six strains of streptococci which did not ferment lactose and had been isolated at various times from faeces of nine horses in the same stable, and fourteen similar isolates supplied by Professor H. W. Seeley (Cornell University) were examined. They all conformed to the descriptions of *Streptococcus equinus* given by Andrewes & Horder (1906) and Hodge & Sherman (1937). The *S. bovis* strains used were from the collection in this laboratory.

Physiological tests

The nutrient medium for growing organisms for physiological tests and for serological investigations was glucose Lemco broth (%, w/v: Evans peptone, 1; Lab. Lemco, 1; NaCl, 0·5; glucose, 0·5). The temperature of incubation was 37°. For physiological tests the inoculum was one loopful (4·0 mm. diam.) of an 18 hr. culture.

Sugar reactions. Tubes of peptone water (1 %; 5 ml.) containing 0·5–1 % of the

test sugar and bromocresol purple as indicator were inoculated and examined daily for 10 days.

Litmus milk was examined daily for 10 days.

Starch hydrolysis. Starch (0·3 %) nutrient agar plates were streaked with organism and after incubation for 2–3 days flooded with Gram's iodine. A colourless or light brown zone under and around the area of growth was recorded as positive.

Ammonia from arginine. Arginine (0·3 %) broth was examined with Nessler's reagent for the presence of ammonia after 3–4 days.

Tolerance of potassium tellurite. Streaked plates of glucose nutrient agar containing potassium tellurite (0·04 %) were examined after 24 hr. Tolerant streptococci (*Streptococcus faecalis*) grow profusely as black colonies. No growth or a dusty grey growth of small colonies was recorded as negative.

Growth on bile blood agar. Glucose yeast-extract nutrient agar containing 40 % (v/v) Difco Bacto oxgall and 5 % (v/v) horse blood was streaked and the plates examined after incubation for 48 hr.

Growth in presence of 2·0 % *and* 6·5 % NaCl. Glucose Lemco broth containing 2·0 % or 6·5 % NaCl (Analar) was inoculated and examined after 24 hr.

Other physiological tests were those used by Shattock (1949) and Sharpe & Shattock (1952).

Serological methods

Preparation of group-specific antisera in rabbits. The technique of Jones & Shattock (1960) was used.

Preparation of antigen extracts. Extracts were prepared by using 0·05 M-HCl (Lancefield, 1933), formamide (Fuller, 1938), alkali (Elliott, 1960). HCl extracts were concentrated by ethanol precipitation (Shattock, 1949).

Gel-diffusion. The method of Mansi (1958) was used. Slides bearing the gel were stored at room temperature in Petri dishes containing moist cottonwool to prevent the gel drying. Results were read after 2–3 hr. This method was compared with the plate method (Mansi, 1957) which gave similar results.

Absorption tests were made as described by Shattock (1949).

Preparation of cell walls. Cell walls were prepared by the method of Cummins & Harris (1956) but without pepsin digestion (Jones & Shattock, 1960).

RESULTS

The serological grouping of Streptococcus equinus

HCl extracts of 48 of the 50 strains of *Streptococcus equinus* examined gave strong precipitin reactions with group D antisera; in most cases it was not necessary to concentrate the extracts by ethanol precipitation; the two apparently aberrant strains are discussed below. All the extracts tested (31) by slide gel-diffusion against group D antisera gave lines of precipitate. They also showed patterns of identity with HCl extracts of accepted group D strains (*S. faecalis* 775; *S. durans* 98 D).

A potent group D antiserum was prepared by using *Streptococcus equinus* strain CN 1 for immunization. This antiserum reacted strongly with HCl extracts of group D strains in the precipitin-ring test and in slide gel-diffusion tests. Patterns of identity were given by extracts of *S. equinus* strains and of other group D strains with the antiserum prepared from *S. equinus* strain CN 1.

The serological grouping of *Streptococcus equinus* strains as belonging to group D was confirmed by reciprocal absorption tests. The group D antibody in *S. equinus* strain CN 1 antiserum was completely absorbed by *S. durans* strain 98 D organisms; similarly, *S. equinus* strain CN 1 organisms completely removed group antibody from the group D antiserum prepared with *S. durans* strain 98 D.

Jones & Shattock (1960) showed that the group D antigen in representative strains of all species of group D streptococci examined was not located in the cell wall but was in the cell contents. Isolations of cell walls were made from two strains of *Streptococcus equinus* (CN 1, T 1). HCl extracts of separated cell walls gave no reaction with group D antisera. The cell contents fraction, however, gave a strong precipitin-ring reaction and lines of precipitate in gel-diffusion tests. The cell contents fraction acidified with HCl to 0·05 M, heated in a boiling-water bath for 5 min. and neutralized, still gave strong reactions with group D antisera in precipitin-ring and gel-diffusion tests.

The HCl extract of cell walls of *Streptococcus equinus* strain CN 1 reacted with the homologous antiserum, indicating that the type antigen was located in the cell wall. Thus the locations of antigens in *S. equinus* are similar to those in other species of group D streptococci (Elliott, 1960).

The two aberrant strains of *Streptococcus equinus* referred to above (24 A, 34 A obtained from Professor H. W. Seeley) were indistinguishable physiologically from the rest of the group. HCl extracts of these strains even after ethanol concentration gave no precipitates with group D antisera. Formamide extracts (Fuller, 1938) gave weak reactions in precipitin-ring and gel-diffusion tests. Because it has been shown that the group D antigen is located in the cell contents (Jones & Shattock, 1960; Elliott, 1960) preparations of cell contents of these two aberrant strains obtained after breaking (Mickle disintegrator) the organisms were tested against group D antisera. Both preparations of cell contents gave strong reactions in precipitin-ring and gel-diffusion tests. Moreover, the precipitates in gel-diffusion test gave patterns of identity with HCl extracts of group D species *Streptococcus faecalis* (strain 775) and *S. faecalis* var. *liquefaciens* (strain Elv. 2025). It therefore appears that the group D antigen present in these two apparently aberrant strains is not extractable from whole organisms by hydrochloric acid. Precipitin-ring reactions were, however, obtained with alkaline extracts (Elliott, 1960) of whole organisms of these two strains when tested against the same group D antisera as used in the previous tests. The alkaline extract of only one of these strains (34 A) gave a line of precipitate in a gel-diffusion test and it showed a pattern of identity.

Comparison of physiological character of Streptococcus equinus *and* S. bovis

In many respects *Streptococcus equinus* resembles *S. bovis*; some workers have questioned the validity of separating the two species (Seeley & Dain, 1960). For this reason we compared the physiological reactions of our 50 strains of *S. equinus* with 43 strains of *S. bovis*. The results are given in Table 1 where they are compared with the characters of *S. equinus* as reported by Hodge & Sherman (1937) and with 95 starch-hydrolysing streptococci conforming to the definition of *S. bovis* described by Seeley & Dain (1960).

Streptococcus equinus characteristically does not ferment lactose; this is a character used to distinguish it from *S. bovis*, which does. Seeley & Dain (1960) pointed out

that this is not a strong reason for separating these two species. Although other physiological reactions may not clearly distinguish between them, we feel that when the physiological reactions are viewed as a whole there is still sufficient reason for retaining the two species. Apart from the separation over lactose fermentation, most of our strains of *S. equinus* did not ferment trehalose, raffinose or inulin, nor hydrolyse

Table 1. *Physiological reactions of* Streptococcus equinus *and* S. bovis

	S. equinus 50 cultures	*S. equinus* Hodge & Sherman (1937), 72 cultures	*S. bovis*, 43 cultures	Starch-hydrolysing streptococci, Seeley & Dain (1960), 95 cultures
Grow at 10°	2	0	0	.
Grow at 45°	96	100	100	95
Resist 60° for 30 min.	2	14	9	.
Resist 60° for 15 min.	12	.	.	.
Grow at pH 9·6	0	.	0	.
NH_3 from arginine	0	.	0	3
Grow in 6·5 % NaCl	0	.	0	0
Grow in 2·0 % NaCl	94	.	.	99*
Grow on 40 % bile	100	.	100	100
No change in litmus milk	100	100	0	3
Tellurite (0·04 %) tolerant	0	.	0	.
Hydrolyse starch	0†	0	95	100
Hydrolyse aesculin	90	100	100	99
Ferment: Arabinose (L+)	9	0	50	48
Xylose	0	0	.	0
Lactose	0	0	100	99
Sucrose	80	92	91	100
Trehalose	12	.	33	68
Raffinose	6	6	82	95
Inulin	12	31	89	96
Mannitol	0	0	13	12
Sorbitol	0	0	0	0
Salicin	100	88	98	.
β-Haemolysis	0	0	0	6‡

* 2·5 % NaCl. † 60 % showed slight starch hydrolysis. ‡ Under CO_2.

Table 2. *Sugar reactions* of Streptococcus equinus *and* S. bovis

	Sugar reaction patterns															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Mannitol	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Trehalose	+	+	−	+	+	−	−	−	−	+	+	−	−	−	−	−
Arabinose	+	+	+	+	−	+	+	−	−	−	−	+	−	−	−	−
Raffinose	+	+	+	−	+	+	−	+	+	−	−	−	−	+	−	−
Inulin	+	+	+	+	+	−	+	−	+	+	−	−	+	−	−	−
Sucrose	+	+	+	+	+	+	−	+	+	+	+	+	+	−	+	−
	No. of strains showing given pattern															
S. equinus (50 strains)	0	0	0	0	0	0	0	0	2	1	5	4	3	1	25	9
S. bovis (43 strains)	4	3	8	2	4	1	4	3	13	1	0	0	0	0	0	0

\+ = acid reaction; − = no reaction.

starch strongly. In contrast, no strain of *S. bovis* was negative in all these tests. An analysis of the sugar reactions of our *S. equinus* and *S. bovis* strains is given in Table 2. The *S. bovis* strains are all in sugar reaction patterns 1 to 10; all the *S. equinus* strains fall into patterns 9 to 16. There is slight overlap of the two species in patterns 9 and 10. Dunican & Seeley (1962) reported that when *S. equinus* hydrolysed starch this differed markedly from starch hydrolysis by *S. bovis: S. equinus* hydrolysed starch (but not to reducing sugar) only in the presence of an easily fermentable carbohydrate. The reaction in litmus milk is also a distinguishing character: as would be expected *S. equinus*, being unable to ferment lactose, gives no reaction in litmus milk whereas *S. bovis* produces acid, though this reaction may be slow (several days) to develop. Thus *S. equinus* and *S. bovis* form fairly distinct physiological groups. There is, however, a spectrum of physiological characters between what might be called typical members of each species. A similar picture is seen in the relationship of some other group D species, for example *S. faecium* and *S. durans* (Lake, Deibel & Niven, 1957; Shattock, 1962).

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