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The Transmissible Nature of the Genetic Factor in *Escherichia coli* that Controls Enterotoxin Production

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SUMMARY

A genetic factor (designated Ent) responsible for enterotoxin production in six of 51 enterotoxigenic strains of *Escherichia coli* of porcine origin could be transmitted to other strains of *E. coli* and to *Salmonella typhimurium* and *S. choleraesuis* by conjugation in mixed culture. A high proportion of organisms of the recipient strains in these cultures was found to have accepted Ent, which was probably a plasmid. The possession of Ent by an organism was a stable characteristic; no organisms were found to have lost it during either laboratory cultivation, acriflavine treatment, or residence in the alimentary tract of pigs and mice. Ent could be transmitted independently of F, R, Hly and K88 factors; the transfer factor responsible for its transmission closely resembled F and the transfer factors of *fi* + R factors and Hly. Ent$^+$ organisms could not be differentiated from Ent$^-$ ones, morphologically, culturally or antigenically. The oral administration of enterotoxin-containing bacteria-free culture fluid of an Ent$^+$ strain of *E. coli* had no apparent ill effect on pigs, piglets and calves; neither did the fluid influence skin permeability. The transmission of Ent to *S. typhimurium* and *S. choleraesuis* did not affect their pathogenicity. Three of four piglets given Ent$^+$ *E. coli* organisms developed diarrhoea; three given Ent$^-$ organisms of the same strain did not.

INTRODUCTION

Smith & Halls (1967*b*, *c*) showed, by using modifications of the method of De & Chatterje (1953), that, with one exception, the serotypes of *Escherichia coli* associated with enteric disease in the pig dilated ligated segments of pig intestine, while other strains did not do so. They also showed that the factor which determined whether or not a strain produced dilatation was ability to produce an enterotoxin. For a strain to produce diarrhoea in the animal, however, it also had to be able to proliferate in the anterior small intestine. The exceptional serotype, O139:K82, is more commonly associated with oedema disease than with diarrhoea; only one of the 23 strains of this serotype examined by Smith & Halls (1967*b*) was positive in the ligated intestine test.

In view of the transmissibility of α haemolysin production in *Escherichia coli* (Smith & Halls, 1967*a*), it was thought that perhaps a transmissible genetic factor might also be responsible for enterotoxin production in the one O139:K82 strain able to produce it. Experiments indicated that this was indeed the case. The results of these experiments and others in which this genetic factor, termed Ent, was transmitted from enterotoxigenic strains of *E. coli* to other strains of this species as well as to

Salmonella typhimurium and *S. choleraesuis* are described in this paper. So are pathogenicity tests with Ent$^+$ and Ent$^-$ organisms and their cell-free culture fluids.

METHODS

Transfer of ability to produce enterotoxin. The method finally adopted to transfer enterotoxin-producing ability was the following. Nutrient broth (Oxoid, no. 2) in 10 ml. amounts was seeded with 0·02 ml. of a 24 hr broth culture of an enterotoxigenic strain of *Escherichia coli* and with 0·02 ml. of a similar culture of a streptomycin-resistant mutant of another strain. The former is referred to as the prospective donor and the latter as the prospective recipient strain. The mixed culture was incubated at 37° for 48 hr and then 0·2 ml. was inoculated into 10 ml. of broth containing streptomycin sulphate 50 μg./ml. After incubation at 37° for 24 hr, 0·1 ml. of this material was inoculated into another 10 ml. of the streptomycin-containing broth. This was incubated similarly and then subcultured in 0·02 ml. amount into 10 ml. of plain broth. After incubation at 37° for 24 hr, this culture, referred to as the passaged mating culture, was inoculated on to a suitable solid medium to confirm, as far as possible, that it contained only organisms of the prospective recipient strain. Whenever it was necessary to distinguish prospective recipient from donor strains, their growth characteristics, drug sensitivity, ability to produce haemolysin and, always, antigenic structure were utilized. If the results of these tests were satisfactory, the passaged mating culture, in 1·0 ml. amount and after the addition of 50 μg. streptomycin sulphate, was submitted to the ligated intestine test in a pig. If it produced dilatation it was subjected to two serial single-colony isolations on MacConkey agar and several single colony isolates were again examined by the ligated intestine test. Individual colonies were tested until at least one dilatation-positive culture was obtained. After confirming once again that the bacteria were those of the recipient strain, a representative number of dilatation-positive cultures, designated Ent$^+$, and dilatation-negative cultures, designated Ent$^-$, from each positive mating culture were freeze-dried and also stored at 5° on Dorset egg medium.

The above procedure was varied occasionally. For example, when the prospective donor strain was itself streptomycin-resistant, then an ampicillin-resistant or nalidixic acid-resistant mutant of the prospective recipient strain was used. The mating culture in this case was passaged through broth containing ampicillin or nalidixic acid, 30 μg./ml., instead of streptomycin. When the prospective recipient and donor strains had the same antigenic structure, a mutant of the recipient resistant to streptomycin and nalidixic acid was used for mating, the mating culture being passaged through broth containing streptomycin only. Very occasionally, a fully drug-sensitive prospective recipient strain was used. When this was a Salmonella the mating culture was passaged through selenite medium instead of drug-containing broth; when it was an *Escherichia coli*, colonies of the prospective recipient strain isolated from the passaged mating culture were tested directly in the ligated intestine, for it was useless to test the passaged mating culture since it would also contain the donor. With some positive passaged mating cultures, colonies were tested first in pools of five; the constituent colonies of any pool giving a positive reaction were then tested separately.

Cell-free enterotoxin-containing culture fluids. These were prepared by the methods of Smith & Halls (1967*c*) using either soft agar or liquid medium. After the culture fluids were separated from the bacteria by centrifugation they were held at 65° for

10 min. to kill the few bacteria that remained, and neomycin sulphate, 100 μg./ml. was added, after which they were tested for enterotoxicity by injection in 15 ml. amounts into ligated segments of pig intestine.

Ligated intestinal preparations. The technique of performing ligated intestinal tests on different species of animals has been described fully elsewhere (Smith & Halls, 1967*b*). Unless otherwise stated, all the intestinal preparations in the present studies were made in pigs. Briefly, weaned pigs that had been starved for 24 hr were anaesthetized and their small intestine, beginning 1 m. from the pylorus and ending 3 m. from the caecum, was divided into segments 10–12 cm. long by string ligatures. Alternate segments were injected with the materials under test and the intermediate ones left as uninoculated controls. It was usually possible to perform 50 tests in each pig. The abdomen was then closed and the pigs allowed to recover from anaesthesia. They were killed 24 hr later and the presence or absence of dilatation of the segments and the volume and character of the fluid they contained was recorded. Since the length of the segments was approximately constant, e.g. ± 25 %, the volume of fluid recovered served as a measure of the severity of the reaction. In assessing this, however, the region of the intestine in which a test was made had to be taken into consideration because the anterior portion is more susceptible to dilatation than the posterior portion, susceptibility decreasing gradually from anterior to posterior. For this reason, materials to be compared were always tested in segments close to each other.

Consistent results are obtained when strains of *Escherichia coli* are tested by the procedure outlined above and positive segments are easily differentiated from negative ones (Smith & Halls, 1967*b*, *c*). Positive segments in the anterior test region contain 20–70 ml. of exudate and in the posterior test region seldom less than 10 ml.; by contrast negative segments contain 0·2–0·5 ml. of red amorphous semi-solid material. The anterior region was always used for testing cell-free culture fluids, the less reactive posterior region being unreliable for this purpose. Bacteria-free fluids from non-enterotoxigenic strains are usually completely absorbed from the segments into which they have been injected. Sometimes, however, a few ml. of thick mucoid fluid remained. By contrast, segments injected with culture fluids of enterotoxigenic strains were strongly dilated and might contain as much as 120 ml. exudate.

As mentioned previously, streptomycin was added to broth cultures of streptomycin-resistant organisms, and neomycin to all bacteria-free culture fluids, immediately before they were tested in the ligated intestine. The purpose of this was to suppress the multiplication of drug-sensitive *Escherichia coli* that might already be present in the segments; very occasionally dilatation of a control uninoculated segment occurred due to the multiplication in it of enterotoxigenic *E. coli*. Whenever a passaged mating culture or isolates from it were being tested, the corresponding prospective donor and recipient strains were always injected into nearby segments.

Detection of R and Hly factors. The presence of transmissible R factors (Watanabe, 1963) was detected by the methods of Smith (1966), and the presence of Hly factors by the methods of Smith & Halls (1967*a*).

The fi character of Ent. This was determined by introducing the Ent factor into an F^+ culture of *Escherichia coli* K12 and then examining it for visible lysis by phage MS2 (Davis, Strauss & Sinsheimer, 1961). The validity of this test has been demonstrated by Meynell & Datta (1966).

Skin permeability. The effect of bacteria-free culture fluids on skin permeability was examined by the method of Craig (1965). The fluids were injected intradermally in 0·1 ml. into the shaven back of rabbits and into the ventral abdominal wall of pigs and calves. The Pontamine sky blue 6XB, 0·12 ml. of a 5% solution/100 g., was given intravenously 24 hr later.

RESULTS

The incidence of transmissible enterotoxin factor (Ent) amongst enterotoxigenic strains of Escherichia coli

Donors. Following the initial observation that strain P3, the only O139:K82 strain of *Escherichia coli* in our original collection to produce enterotoxin, could transfer this property to non-enterotoxigenic strains, a further 50 strains that had been incriminated in different outbreaks of diarrhoea in pigs were examined to determine whether their enterotoxigenic properties were also transmissible. Five of the 50 were positive. Two of the five, P18 and P103, had the antigenic formula O8:K87,88ab and two others, P16 and P19, the formula O9:K9; the latter two were the only O9:K9 strains examined. The remaining strain, P255, was an O139:K82 strain that did not belong to our original collection. Consequently, P255 and P3 were the only two of twenty-four O139:K82 strains that produced enterotoxin and with both strains its production was transmissible.

In strains of the following serotypes enterotoxin production could not be shown to be transmissible, O141:K85ac (4 strains), O141:K85ab → 85ac (9 strains), O141:K85ab,88ab (8 strains), O8:K87, 88ab (1 strain), O8:K87, 88ac (4 strains), O147:K89,88ac (3 strains), O45:K?,88ac (2 strains) and O138: K81 (8 strains). Neither was enterotoxin production transmissible by 6 strains of the Abbotstown type, a type not yet been given an internationally accepted formula.

Recipients. Every strain of *Escherichia coli* examined for ability to act as recipient was tested by using strain P3 as prospective donor of the Ent factor. Many strains were also tested with P16 and P103 as donors and a few with P18 and P19. The factor was transmitted to the following strains: (1) K12F$^-$ and F$^+$. (2) Five non-pathogenic strains isolated from the faeces of healthy pigs in different herds. (3) An O139:K82 strain, P104, incriminated in an outbreak of oedema disease. (4) An O18:K? strain, F11, responsible for an outbreak of bacteriaemia in broiler fowl. (5) An O26:K60:H- strain, H1, from a case of neonatal diarrhoea in a human being. This strain had consistently dilated rabbit intestine (see Smith & Halls, 1967*b*). Only after mixed culture with P3, however, did it dilate pig intestine. The O139:K82 strain, P104, was probably the best recipient; the Ent factor was readily transmitted to it from all six donors. Dilatation of pig intestine was also achieved with bacteria-free culture fluids of strains P104, F11, H1, K12F$^-$ and K12F$^+$ into which Ent had been introduced.

Figure 1 illustrates a positive and a negative result obtained during the testing of passaged mating cultures in ligated pig intestine.

Despite repeated tests with one or more of the six donors it was impossible to transmit Ent to 14 strains of *Escherichia coli.* These included three non-pathogenic strains isolated from healthy pigs in different herds. They also included four O139:K82 strains, strains of the same serotype as the good recipient, P104. These strains had been tested using each of 3 known donors, P3, P16 and P103 on several occasions. Ten

passaged mating cultures involving one of the four and P103 all yielded negative results. By contrast, 11 of 20 such cultures of P103 and P104 performed at the same time had yielded positive results.

The transmission of Ent from Escherichia coli to other bacterial species

Attempts were made to transmit the Ent factor from the *Escherichia coli* strains P3, P16, P19 and P103 to 10 strains of *Salmonella typhimurium*, 10 strains of *Shigella flexneri*, two strains each of *S. dublin*, *Proteus vulgaris* and *P. morganii*, and one

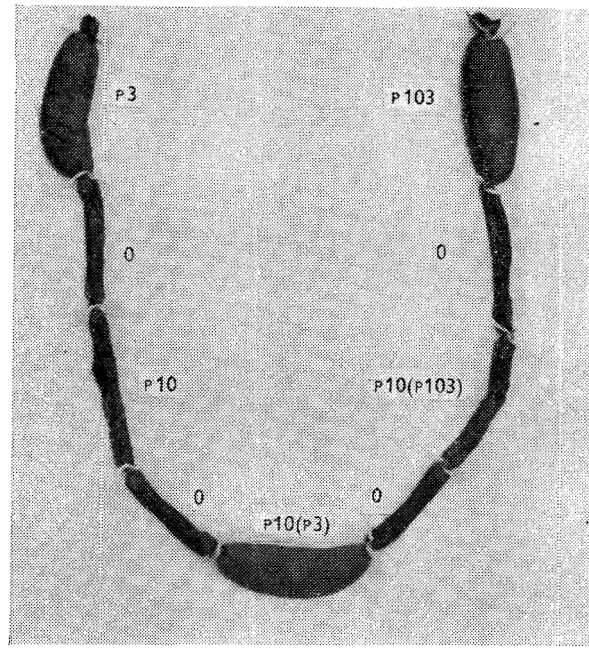

Fig. 1. Ligated segments of pig intestine inoculated with cultures of *Escherichia coli*. The segments are identified according to the strains used for inoculating them. P3 and P103 = enterotoxigenic strains. P10 = non-enterotoxigenic strain. P10 (P3) and P10 (P103) = passaged mating cultures of P10 in which P3 and P103 had been employed as prospective donors. The ability to produce enterotoxin has been transferred to P10 from P3 but not from P103. O, uninoculated segments. × ½.

strain each of *S. choleraesuis*, *Shigella sonnei*, *Klebsiella aerogenes* and *Pseudomonas aeruginosa*. Transmission was achieved to three strains of *S. typhimurium*, and to the one strain of *S. choleraesuis*, but not to any of the other prospective recipients. It was transmitted to one strain of *S. typhimurium* on 11 of 14 occasions from *E. coli* P3, P16 and P19; the result of testing this strain before and after the introduction of Ent is illustrated in Fig. 2. By contrast seven passaged mating cultures of this strain with P103 as the prospective donor yielded negative results when tested in ligated pig intestine. After transfer to *E. coli* P104 from *S. typhimurium* Ent$^+$ and *E. coli* F11 Ent$^+$, the Ent factor retained its transmissible nature.

Since we were successful in transferring Ent to *Salmonella typhimurium*, we looked

for naturally occurring strains of this organism that would give a positive reaction in the ligated intestine test. Of 37 epidemiologically unrelated strains, 36 were negative. But one strain, 1652, produced some degree of dilatation which was less than that produced by strains of *S. typhimurium* to which Ent had been transferred from *Escherichia coli*. For example, the volume of exudate provoked by strain 1652 and a strain of *S. typhimurium* Ent$^+$ in adjacent test segments in four different regions

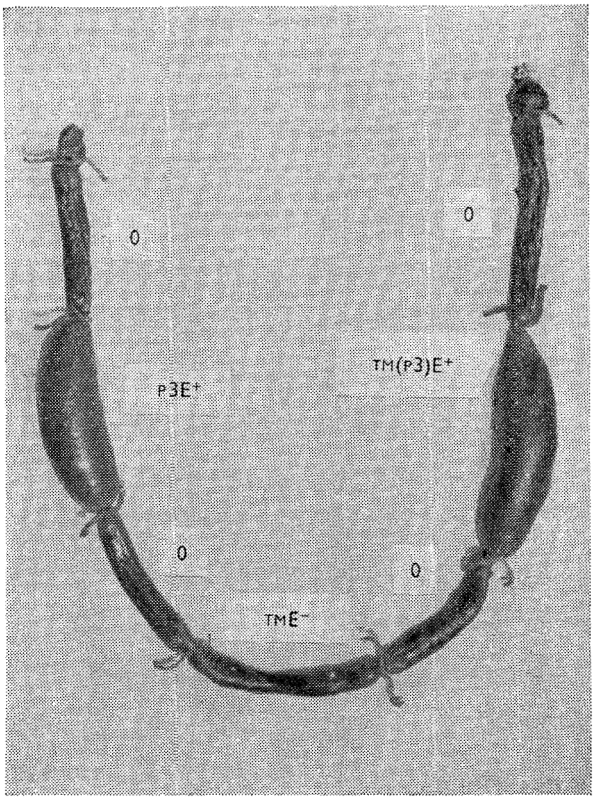

Fig. 2. Ligated segments of pig intestine inoculated with cultures of *Salmonella typhimurium* and *Escherichia coli*. The segments are identified according to the strains used for inoculating them. P3E$^+$ = enterotoxigenic strain of *E. coli*. TME$^-$ = non-enterotoxigenic strain of *S. typhimurium*. TM(P3)E$^+$ = enterotoxigenic strain of *S. typhimurium* obtained by mating P3 with TME$^-$. O = uninoculated segments. × ½.

of the small intestine of a pig commencing in the more susceptible anterior part and ending in the less susceptible posterior part were, respectively, 19 and 30, 12 and 33, 0 and 29, and 0 and 12. It was not possible to transmit the ability to produce dilatation from 1652 to *S. typhimurium* or to *E. coli*.

The extent of Ent transfer in passaged mating cultures

From every passaged mating culture which dilated ligated pig intestine at least one colony of the recipient strain that had acquired Ent was obtained. The results of examining individual colonies from passaged mating cultures are shown in Table 1.

The incidence of Ent$^+$ organisms in these cultures varied from 2 to 87%. Only five colonies of *Escherichia coli* K12F$^-$ were tested after mating with *E. coli* P3; all produced dilatation.

Before classifying a colony as Ent$^+$ or Ent$^-$ in the tests referred to above, it was tested in the ligated intestine of at least three pigs; consistent results were obtained. Control cultures of the prospective donor and recipient strains were always tested together with the passaged mating cultures and colonies obtained from them and they, too, yielded consistent results. Two of the good recipient strains, *Escherichia coli* P104 and a *Salmonella typhimurium* strain, were tested 70 and 35 times, respectively, during the present studies; they failed to dilate ligated pig intestine on every occasion. Cultures of 100 colonies from each of these two strains and also from each of another two strains of *E. coli*, P30 and F11, that were also capable of accepting Ent, were tested in pools of five in 10 pigs. A further 25 colonies obtained from the *S. typhimurium* strain were tested singly. Dilatation was never produced.

Table 1. *The incidence of Ent$^+$ recipient organisms in passaged mating cultures*

Passaged mating culture		Colonies of recipient strain obtained from passaged mating culture	
Recipient	Donor	No. tested	% Ent$^+$
E. coli P104	*E. coli* P16	10	70
E. coli P104	*E. coli* P18	15	40
E. coli P104	*E. coli* P103	20	50
E. coli P104	*E. coli* P103	18	50
E. coli P20	*E. coli* P3	20	80
E. coli P30	*E. coli* P3	50	6
E. coli P30	*E. coli* P16	20	5
E. coli P66	*E. coli* P103	15	53
E. coli F11	*E. coli* P3	25	12
E. coli H1	*E. coli* P3	50	2
S. typhimurium strain 1	*E. coli* P3	15	67
S. typhimurium strain 1	*E. coli* P3	18	33
S. typhimurium strain 1	*E. coli* P3	18	22
S. typhimurium strain 1	*E. coli* P16	15	87
S. typhimurium strain 1	*E. coli* P19	10	60
S. typhimurium strain 1	*E. coli* P19	10	50
S. typhimurium strain 2	*E. coli* P3	25	36
S. choleraesuis	*E. coli* P3	10	60

P20, P30 and P66 were non-pathogenic strains of *E. coli* isolated from the faeces of healthy pigs.

The comparative dilating ability of different bacterial strains into which Ent had been introduced

Although detailed studies were not made, the general impression gained was that the degree of dilatation produced by a given strain into which Ent had been introduced was more a function of that strain than of the Ent factor or the strain which had donated it. For example, there was no important difference between the amount of fluid that accumulated in segments of pig intestine which had been injected with strains of *Escherichia coli* P104 into which Ent had been introduced from strains P3, P16, P18, P19, P103 or P255. The amount was usually in the region of 20 ml. when

tests were made in the anterior small intestine. Similar volumes were usually provoked by P3, the donor strain of the same serotype as P104. Greater volumes were commonly provoked by the other donor strains P16, P18 and P103; P255 was not tested often enough to permit any conclusions being drawn. In contrast to the Ent$^+$ derivatives of P104, those of *Salmonella typhimurium* that had received the factor from P3, P16 or P19 produced much greater dilatation, the volumes provoked in the anterior intestine being 30–50 ml. irrespective of the donor strain involved. The *E. coli* strains K12F$^-$ Ent$^+$ and K12F$^+$Ent$^+$ were the poorest dilators. These strains usually had to be injected in greater numbers than other strains to produce dilatation, and then the volume of exudate provoked was only 3–10 ml. The reason for this was probably poor survival of the bacteria in the ligated segments, probably because K12 strains have been maintained in the laboratory away from their natural habitat for many years. Support for this view was provided by the observation that bacteria-free culture fluids of these strains produced dilatation of ligated segments as strongly as culture fluids of the other Ent$^+$ strains (Fig. 3). As an example of the dilatation produced by culture fluids, when culture fluids of P104 into which Ent had been introduced from P3 were tested in seven segments in one pig, the volume of exudate that accumulated in these segments was 40–90 ml. (median 50 ml.); the volumes in segments injected with culture fluids of P104 itself was 2–9 ml. (median 5 ml.).

The stability of Ent in bacteria; the effect of acriflavine

All the *Escherichia coli* and Salmonella strains to which Ent had been transmitted were maintained on Dorset egg medium at 5°. Between the time of inoculation and the completion of the work described in this paper (5–12 months) they were examined on many occasions. Their ability to dilate ligated pig intestine remained constant. Apart from periodic subculture, five of the six wild strains of *E. coli* from which Ent was transmitted had been maintained on Dorset egg medium for 6 years before they were examined.

Three strains of *Escherichia coli*, K12F$^-$, H1 and F11, and one strain of *Salmonella typhimurium* to which Ent had been transmitted, were passaged daily for 10 days in nutrient broth at 37° by using small inocula transferred with the tip of a platinum wire. Five colonies from the final subculture of each strain were tested in ligated pig intestine; all produced dilatation.

No loss of Ent was detected from *Escherichia coli* or Salmonella organisms during residence in experimentally infected mice and pigs. The period between infection and recovery of *S. typhimurium* Ent$^+$ and *S. choleraesuis* Ent$^+$ in mice and/or pigs was 5–17 days. In the case of *E. coli* P104 Ent$^+$ given to two pigs by mouth it was 63 days. *Escherichia coli* Ent$^-$ and Salmonella given to pigs and/or mice did not acquire the ability to produce enterotoxin.

Strains of *Salmonella typhimurium* and *Escherichia coli* P104 to which Ent had been transmitted from *E. coli* P3 were incubated at 37° for 24 hr in nutrient broth containing acriflavine at a concentration which had a marked depressant effect on their growth rate. Twenty-five colonies from each of the resulting cultures were tested in ligated pig intestine; none had lost the ability to produce dilatation.

The significance of culture filtrates, phages, colicines, F, R, Hly and K88 factors in Ent transfer

Culture filtrates. Ten passaged mating cultures were prepared in the usual manner except that filtrates, passed through a membrane of average pore diameter 250 mμ in 5–10 ml. amounts, of 24 hr nutrient broth cultures of the donor strains of *Escherichia coli*, P3, P16, P18, P19, P103 and P255, were used instead of the donor organisms themselves. The prospective recipient strain used in each mating culture was one or other of four strains of *E. coli*, P104, P30, H1 and F11, or a *Salmonella typhimurium* strain of proven recipient ability. None of the 10 cultures dilated ligated pig intestine.

Phages and colicines. Cultures of the six strains that were able to transmit Ent and of six whose enterotoxigenic character was not transmissible were tested for phage or colicine production with cultures of six strains that were able to receive Ent and six that were not. A strain of *Salmonella typhimurium* was included amongst the strains in each of the last two categories; the remaining 22 strains were of *Escherichia coli.* Five strains before and after they had received Ent were also tested against each other. Apart from the fact that one of the six strains that could not act as a recipient exhibited strong colicine action against 11 of the 12 strains which had been tested as donors, no phage or colicine activity was observed. Neither was any activity between the donor strains and the four strains of *E. coli* that were of similar antigenic structure to P104, the good recipient, but were unable to receive Ent.

F factor. As mentioned previously, Ent was transmitted to an *Escherichia coli* K12F$^-$ strain and to a K12F$^+$ strain. No more difficulty was experienced in transmitting it to the K12F$^+$ strain than to the K12F$^-$ strain. The K12F$^+$ strain was no longer macroscopically lysed by the F specific phage MS2 after the introduction of Ent indicating that Ent was an *fi*+ factor (fertility inhibiting; Watanabe *et al.* 1964). In the same way as *fi*+ R factors (Meynell & Datta, 1966) and the Hly factor, Ent itself carried the gene for production of an F-like pilus, because F-specific phage, which uses the pilus as receptor, was able to multiply in cultures of K12F$^-$Ent$^+$ bacteria (Dr Elinor Meynell, personal communication).

R factors. Of the six strains of *Escherichia coli* shown to be able to transmit Ent, one, P18, contained R factors. The other five strains, as well as the 10 strains of *E. coli* and three Salmonella strains that were able to receive Ent, did not. Of four Ent$^+$ colonies of *E. coli* P104 obtained from a passaged mating culture in which P18 was the donor, all four contained R factors. Of four Ent$^-$ colonies from the same mating culture, two contained R factors and two did not.

Different R factors in four strains of *Escherichia coli* were transmitted to six strains of *E. coli* and one strain of *Salmonella typhimurium* into which Ent had already been introduced and into their Ent$^-$ parent strains. There was no obvious difference in the rate of transmission of R factors into the Ent$^+$ and Ent$^-$ form of each of the seven strains. Dr E. S. Anderson (Enteric Reference Laboratory, Colindale, London) examined a *S. typhimurium* strain and the K12F$^-$ strain for the presence of a transfer factor by his resistance determinant mobilization test (Anderson, 1965) before and after Ent had been transmitted to them. No transfer factor could be demonstrated in the two parent strains, which were Ent$^-$, whereas both Ent$^+$ derivatives carried one. As this test depends on mobilization of a determinant for streptomycin and sulphonamide resistance, its success indicates that the transfer factor concerned

can carry genetic determinants for both enterotoxin production and drug resistance.

Hly factor. Three of the six strains of *Escherichia coli* that were able to transmit Ent produced α haemolysin and so did one of the 10 strains of *E. coli* that were able to receive it. In none of these four strains was haemolysin production transmissible, as with the Hly factor (Smith & Halls, 1967*a*). All the 45 strains from which Ent was not transmitted produced α haemolysin, but in only seven strains was its production shown to be controlled by the transmissible Hly factor. Five of these seven also carried R factors. R factors alone were demonstrated in another eight of the 45 strains. One of the Hly$^+$ R$^+$ strains also possessed the K88 factor (see below).

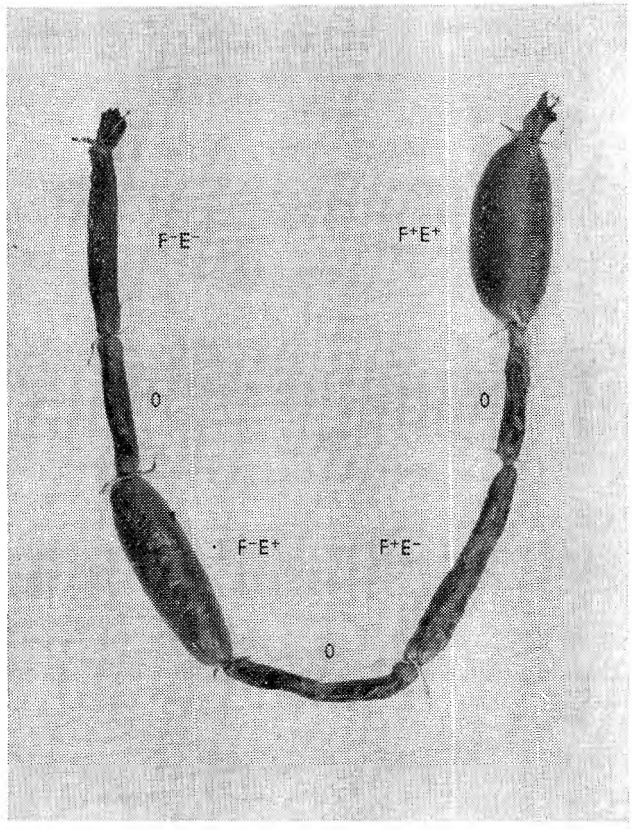

Fig. 3. Ligated segments of pig intestine inoculated with bacteria-free culture fluids of *Escherichia coli*. The segments are identified according to the strains from which the culture fluids were obtained. F$^-$ E$^-$ and F$^+$ E$^-$ = non-enterotoxigenic strains K12F$^-$ and K12F$^+$. F$^-$ E$^+$ and F$^+$E$^+$ = enterotoxigenic strains of K12F$^-$ and F$^+$ obtained by mating with the enterotoxigenic strain P3. O = uninoculated segments. $\times \frac{1}{2}$.

The Hly factor was introduced without difficulty from two Hly$^+$ strains of *Escherichia coli* into four Ent$^+$ strains of *E. coli*, two of these recipient strains being derived from *E. coli* K12F$^-$ and F$^+$. During these mating procedures, R factors present in one of these two Hly$^+$ donor strains was also transmitted to some of the recipient organisms. Some K12F$^+$ organisms, for example, contained as a result R, Hly and Ent factors.

The two Hly$^+$ donor strains were also enterotoxigenic but passaged mating cultures of strains of *E. coli* able to receive Ent with these two strains as prospective donors did not dilate ligated pig intestine.

K88 factor. Four different strains of *Escherichia coli* into which the genetic elements controlling the production of K88 antigen (Ørskov & Ørskov, 1966) had been transmitted were tested in ligated pig intestine. One of these K88-containing strains had been prepared in this laboratory; the other three were received from Dr Ida Ørskov. All four strains failed to cause dilatation. The donor strains used in the preparation of these four strains were all enterotoxigenic, as are many strains of *E. coli* which produce the K88 antigen. Only two of the six strains that were able to transmit Ent (P18, P103) possessed K88 antigen. None of the strains to which they had transmitted Ent were found to have received this antigen.

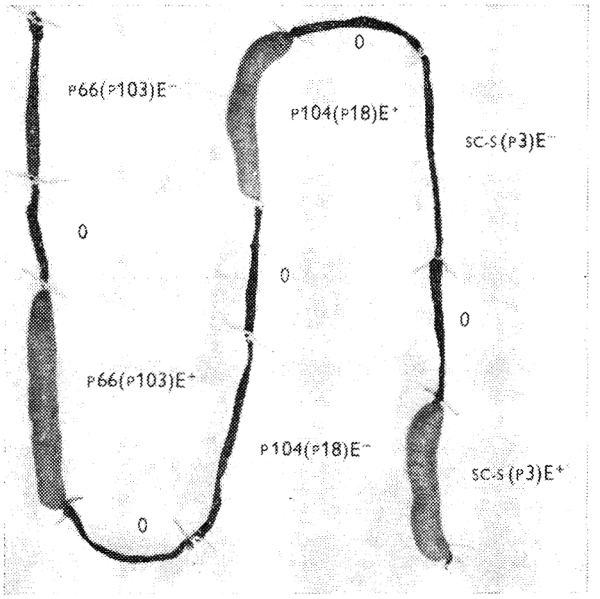

Fig. 4. Ligated segments of calf intestine inoculated with cultures of *Escherichia coli* and *Salmonella choleraesuis*. The segments are identified according to the strains used for inoculating them. P66 (P103) E^+ and E^- = enterotoxigenic and non-enterotoxigenic strains obtained from a mating of the non-enterotoxigenic *E. coli* P66, with the enterotoxigenic *E. coli* P103. P104 (P18) E^+ and E^- = enterotoxigenic and non-enterotoxigenic strains obtained from a mating of the non-enterotoxigenic *E. coli* P104 with the enterotoxigenic *E. coli* P18. SC-S (P3) E^+ and E^- = enterotoxigenic and non-enterotoxigenic strains obtained from a mating of a non-enterotoxigenic strain of *S. cholerae-suis* with the enterotoxigenic *E. coli* P3. O = uninoculated segments. × ⅓.

The effect of Ent$^+$ bacteria on the ligated intestine of calves, rabbits and mice

Although ligated pig intestine was used to determine the Ent character of bacterial cultures in all the studies referred to previously, some cultures were also tested in the ligated intestine of other animal species.

Calves. With two calves, six strains of *Escherichia coli* into which Ent had been introduced were tested and all produced dilatation of intestinal segments. Similar

results were obtained with a *Salmonella typhimurium* Ent$^+$ strain and the *S. choleraesuis* Ent$^+$ strain. A portion of the ligated intestine of one of the two calves is illustrated in Fig. 4.

Rabbits. Two strains of *Salmonella typhimurium* which had received Ent from one or other of two donor strains and the *S. choleraesuis* Ent$^+$ strain failed to dilate ligated intestine, so did the two donor strains concerned, P3 and P16.

Mice. With 10 mice, *Escherichia coli* P104, a strain of *Salmonella typhimurium* and *S. choleraesuis*, all of which had received Ent from *E. coli* P3, and P3 itself, were submitted to the ligated intestine test. Negative results were obtained.

The distinguishing features of Ent$^+$ and Ent$^-$ organisms

Cultures of colonies obtained from dilatation-positive passaged mating cultures and classified as Ent$^+$ or Ent$^-$ according to their reaction in ligated pig intestine were examined to see whether the two kinds of cultures differed in other respects, especially in their pathogenicity. Strain *Escherichia coli* P104 to which Ent had been transmitted from *E. coli* P3 was frequently used in these studies because strains P104 and P3 were both of the same serotype, O139:K82. Another reason for using this strain P104 was the possibility that it might be able to proliferate in the anterior small intestine of the intact pig by virtue of the fact that its parent had been isolated from a case of oedema disease. This would be of major significance in infection studies. All the other strains of *E. coli* to which Ent had been transmitted were non-pathogenic for the pig.

Cultural characteristics. The morphology on ordinary laboratory media of colonies of Ent$^+$ and Ent$^-$ organisms obtained from the same passaged mating culture appeared identical. Their microscopical appearance and their general cultural properties, too, were similar.

Antigenicity. Antisera were prepared in six rabbits by the multiple intravenous injection of live broth cultures of one or other of three different strains of *Escherichia coli* into which Ent had been introduced from *E. coli* P3 or of bacteria-free enterotoxin-containing culture fluids of one or other of these three strains. Each antiserum was then absorbed with suspensions of the corresponding Ent$^-$ organisms. All the absorbed antisera failed to agglutinate suspensions of homologous and heterologous Ent$^+$ organisms, live or heat-treated to destroy their K antigens. The unabsorbed antisera also failed to agglutinate suspensions of Ent$^+$ strains of *E. coli* and Salmonella except those for which they were known to contain O or K antibodies.

No antigen-antibody reactions were detected in plate precipitin tests (Ouchterlony, 1949) between extracts of Ent$^+$ organisms or their enterotoxin-containing culture fluids and the absorbed antisera referred to above.

Antisera were prepared in six pigs by multiple intravenous injection of bacteria-free enterotoxin-containing culture fluids of *Escherichia coli* P3, P16 or P18, three of the wild Ent$^+$ strains, or by the parenteral administration of suspensions of live organisms of these strains. The addition of 10 ml. antiserum to 15 ml. bacteria-free culture fluid of the strain against which each one was prepared and to fluids of other enterotoxigenic strains had no effect on the size of the dilatation the fluids produced in ligated pig intestine.

Pathogenicity of live cultures to piglets. Seven piglets which had been removed from their mother before consuming colostrum were given 40 ml. of sterile cow's

milk, by stomach tube, before they were 4 hr old. Immediately, four were inoculated orally with 5×10^9 viable organisms of *Escherichia coli* P104 to which Ent had been transmitted from *E. coli* P3. The remaining three piglets were inoculated in a similar manner with P104 itself. Subsequently, all piglets were given 40–50 ml. of the cow's milk 3–5 times daily by stomach tube. Of the 4 given the Ent$^+$ strain, one developed severe diarrhoea 11 hr after inoculation, It was killed 9 hr later when still suffering from diarrhoea. The number of viable organisms of the inoculating strain in the contents of its anterior small intestine was, $\log_{10}$ per ml., 7·2. Another developed severe diarrhoea at 30 hr and was killed when near death at 38 hr. The condition of a third piglet in this Ent$^+$ group began to deteriorate rapidly at 40 hr and at 44 hr, when it was killed, it had developed severe diarrhoea. The viable counts of the inoculating strain in the anterior small intestinal contents of both these piglets were relatively low, $\log_{10}$ per ml., 5·3 and 5·2, respectively. No organisms were found in the internal organs of either of these three piglets. The remaining piglet in this group remained healthy as did all three piglets given P104.

Two groups of 4 pigs aged 8 weeks were inoculated orally with approximately 10^{10} viable organisms of *Salmonella choleraesuis*. One group was given an Ent$^+$ strain and the other an Ent$^-$ one, both being obtained from the same mating of a strain of *S. choleraesuis* with *Escherichia coli* P3. The clinical signs subsequently exhibited by both groups were identical. All eight pigs developed a severe diarrhoea, of similar intensity and duration, 3 days after inoculation.

Pathogenicity of live cultures to calves. Two 2-day-old colostrum-fed calves were given, by mouth, 10^{10} viable organisms of *Salmonella typhimurium* Ent$^+$ and another two a similar dose of *S. choleraesuis* Ent$^+$. The four calves died 3–5 days later, the principal lesion in all of them being a severe inflammation of the small intestine with necrosis of the ileum. Diarrhoea was not a prominent clinical sign. The course of the disease was similar to that observed in calves infected with Ent$^-$ forms of these two Salmonella types.

Pathogenicity of live cultures to mice. Ent$^+$ and Ent$^-$ colonies obtained from matings of a *Salmonella typhimurium* strain with *Escherichia coli* P3, P16 and P19 were administered orally in doses of 10^9 viable organisms to groups of mice. The mortality pattern was similar in all groups indicating no difference in virulence between the Ent$^+$ cultures and the Ent$^-$ ones. Diarrhoea was no more common in mice given Ent$^+$ organisms than in mice given Ent$^-$ organisms. The mouse-virulence of *S. choleraesuis* also, was not altered as a result of accepting Ent. Neither was its virulence for rabbits.

Pathogenicity of bacteria-free culture fluids to pigs. Ten piglets were removed from their mothers before they had consumed colostrum and each one given, by stomach tube, 50 ml. of bacteria-free culture fluid of *Escherichia coli* P104 to which Ent had been transmitted from *E. coli* P3. Another 10 piglets were treated similarly except that the fluid they were given had been prepared from an Ent$^-$ strain of P104. The 20 piglets were returned to their mothers $2\frac{1}{2}$ hr later. All remained healthy. So did four 11-week-old pigs that were starved for 48 hr and then given 1000–1500 ml. of the bacteria-free culture fluid of *E. coli* P104 Ent$^+$ by stomach tube.

Pathogenicity of culture fluids to calves. Two 1-day-old calves that had received no food were given 1500 ml. of the bacteria-free culture fluid of *Escherichia coli* P104 Ent$^+$ by mouth. They showed no signs of ill-health as a result.

Pathogenicity of culture fluids to mice. Bacteria-free culture fluids, in 0·5 ml. amounts,

of three strains of *Escherichia coli* into which Ent had been transmitted from *E. coli* P3 were each injected intravenously into 2 mice. All 6 mice remained well.

Skin permeability. No difference was noted in pigs, calves and rabbits between the skin reactions produced by bacteria-free culture fluids of *Escherichia coli* P104 Ent^+ and Ent^-.

DISCUSSION

The results indicate that the ability to produce enterotoxin can be transmitted from some strains of *Escherichia coli* to others and to Salmonella strains and that transmission when it occurs is probably by conjugation. The available evidence suggests that, as in the case of the R, col, K88 and Hly factors, the actual production of enterotoxin in the donor strains is governed by an extrachromosomal genetic element, or plasmid, which has conjugation factor activity. The name Ent is proposed for this element which evidently belongs to the same category of sex factors as F, *fi*+ R factors and Hly which all determine the production of an F-type pilus subject to the same genetic regulation of function (Lawn, Meynell, Meynell & Datta, 1967).

No exclusion of the kind described by Scaife & Gross (1962) to occur between F factors was found to exist between Ent and F, R and Hly factors. They could be carried together in the same bacterium; all four were present in one of our laboratory strains of *Escherichia coli* K12 and a wild strain was found to contain R, Hly and the K88 factor, all transmissible to a K12F^+ strain. When more than one was present in a strain, they were sometimes transmitted together, sometimes separately. Dr E. S. Anderson has utilized the transfer factor of Ent from the K12F^- Ent^+ strain and from one of the *Salmonella typhimurium* Ent^+ strains to mobilize R determinants (Anderson, 1965). It may well be that there is no specific relationship between a transfer factor and a particular plasmid. The transfer factor merely potentiates conjugation without exercising any control on the plasmids that are transmitted during the process.

Although the enterotoxin of many pig strains of *Escherichia coli* appeared to be similar (Smith & Halls, 1967*b*, *c*) most of the strains could not transmit the character. There are several possible reasons for this: the genes may be integrated in the chromosome; if plasmids are responsible, transfer factor activity may be absent; finally, the plasmids may be highly repressed like some R factors (Meynell & Datta, 1966). The only means of identifying an enterotoxigenic strain is the ligated intestine test, which severely limits experiments to distinguish between these possibilities. Not only does it restrict the number of tests that can be performed but the test itself becomes insensitive when enterotoxigenic organisms injected into a ligated segment are greatly outnumbered by non-enterotoxigenic ones (Smith & Halls, 1967*b*). This means that only those passaged mating cultures containing a comparatively high proportion of Ent^+ recipient organisms would be identified; in fact, the lowest proportion found in the present studies was 2%. It is probable then that if suitable techniques had been available more strains could have been identified as donors of their enterotoxin production.

After its introduction into organisms of *Escherichia coli*, *Salmonella typhimurium* and *S. choleraesuis* Ent behaved as a stable character. No organisms were found to have lost it *in vivo* or *in vitro*, even after acriflavine treatment. In view of this stability and the fact that a high proportion of organisms of some of the strains of non-pathogenic *E. coli* isolated from pig faeces that were used as recipients in mating cultures accepted Ent, a substantial degree of transfer might be expected to have occurred

in nature as has happened in the case of R factors; Smith & Halls (1967*b*), did not find this to be so. All the strains of *E. coli* they examined which were not incriminated in outbreaks of diarrhoea in pigs did not dilate pig intestine. It may well be that, unlike the profound influence the widespread use of antibiotics has had on the emergence of R factor-containing bacterial populations, no comparable influences exist to favour the selection of Ent$^+$ organisms. The possession of Ent alone may be of little selective advantage; the selection advantage possessed by enteropathogenic *E. coli* organisms probably resides principally in their ability to proliferate in the anterior small intestine.

The fact that three of the four piglets given *Escherichia coli* P104 into which Ent had been introduced developed diarrhoea, whereas all three given P104 itself remained healthy, supports the view that enterotoxin is responsible for the production of diarrhoea in the intact animal. In two of the piglets that had diarrhoea, however, the numbers of the infecting organisms in the anterior small intestine when they were killed was lower than that usually found in piglets suffering from *E. coli* diarrhoea. In view of this and the fact that the number of animals involved was small the evidence obtained from this experiment is suggestive rather than conclusive. Following its isolation from a case of oedema disease, the P104 strain had been maintained in the laboratory for some 12 years before it was used in the present work; perhaps during this period its ability to proliferate in the small intestine had become impaired. It was not possible to repeat this experiment with more recently isolated non-enterotoxigenic pathogenic strains because of our inability to introduce Ent into any of them. The failure of enterotoxin-containing bacteria-free culture fluids of *E. coli* to produce diarrhoea in the newly born piglets after its administration by mouth is probably explained by the fact that, even in ligated intestine studies, it is only the anterior small intestine that is susceptible to the dilating action of enterotoxin. In the piglets the fluid would not be expected to be retained for long in this anterior region and any fluid disturbance it might cause there would be corrected by absorption in the more posterior region. The situation is quite different from that occurring in ligated intestine studies where the injected enterotoxin-containing fluid is trapped for a long time in an isolated intestinal segment. It is also quite different from that which occurs in *E. coli* infection in the intact animal where enterotoxin is being continuously produced by large numbers of bacteria closely adherent to the mucous membrane.

The introduction of Ent into *Salmonella typhimurium* and *S. choleraesuis* did not enhance their pathogenicity for mice, rabbits, calves and pigs. It would not be expected to do so in mice and rabbits unless the deleterious effect of enterotoxin was not confined to the alimentary tract; the Ent$^+$ Salmonella strains did not dilate the ligated intestine of these two animal species. In calves and pigs the chain of events beginning with the entry of enteropathogenic *Escherichia coli* into the small intestine and ending with the outpouring of fluid into this organ is undoubtedly complex. It would be unlikely to occur as a result of salmonellas entering the alimentary tract purely because they were able to produce *E. coli* enterotoxin. The pathogenesis of infections with enteropathogenic *E. coli* and with Salmonella are markedly different. For example, in the *E. coli* infections the bacteria remain localized in the alimentary tract whereas in Salmonella infections they invade the body. In *E. coli* infections the mucous membrane of the small intestine exhibits no obvious histological changes (Smith & Jones, 1963) whereas in Salmonella infections it is severely inflamed (Smith & Jones,

1967); in the calves used in the present study, extensive necrosis of the mucous membrane of the small intestine had occurred within a few days of their infection with salmonellas. It is therefore not surprising that the diarrhoea in the calves and pigs infected with Ent$^+$ Salmonella strains was no more severe than in calves and pigs infected with Ent$^-$ strains; the mechanism which gives rise to *E. coli* diarrhoea probably could not operate.

It appears that enterotoxin production is yet another characteristic that can be transmitted from bacterium to bacterium. The number of such characteristics continues to grow. Although much remains to be discovered, the point has now been reached at which the transmissible plasmid must be regarded as a normal method of inheritance in some bacterial species, rather than as a rare biological accident.

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Lysis of Non-marine Fungi by Marine Micro-organisms

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SUMMARY

A number of non-marine Oomycetes and a marine-isolated Pythium culture were lysed in natural sea water by marine micro-organisms. A relationship was observed between the rate and extent of lysis and the size of the marine microbial population. Microscopic observations and the accumulation of reducing sugars indicated that extracellular cell wall-lysing enzymes were responsible. Pure cultures of lytic bacteria isolated from the sea yielded data similar to those obtained in natural sea water. The importance of the lytic phenomenon in the determination of the marine mycoflora is discussed.

INTRODUCTION

In the field of marine mycology it is necessary to investigate the biochemical activities of fungi in the oceans before we can understand their ecological significance and their distinct characters. The mycoflora of the oceans has not been studied in enough detail to identify marine-isolated fungi as specific halophiles, salt-adapted species, or non-marine fungi capable of survival in sea water (Johnson & Sparrow, 1961). MacLeod & Onofrey (1963) and ZoBell (1946) showed that some marine bacteria could develop the capacity to grow in the presence of reduced salt concentrations or even in freshwater media. Thus we may ask if some freshwater fungi adapt to marine conditions while others are eradicated by a biological process.

Previous data from our laboratory have shown that a specific marine microflora capable of killing *Escherichia coli* develops when that bacterium is added to sea water (Mitchell, Yankofsky & Jannasch, 1967). The presence in soil of micro-organisms capable of killing non-indigenous fungi by lysing their cell walls is well documented (Mitchell & Alexander, 1963). The present paper describes results obtained when a fungus whose habitat is soil was added to sea water. The fungus *Pythium debaryanum* was used because of the detailed information available about its cell-wall structure (Cooper & Aronson, 1967).

METHODS

In the current study the representative Phycomycete used was *Pythium debaryanum* ATCC9998. Other Oomycetes used were *Saprolegnia diclina*, *Achlya caroliniania*, *Isoachlya luxuriana*, *Thraustotheca clavata* and *Apodachlya brachynema*. Fungal mats were grown in Petri dishes using a potato dextrose broth at 23°, and were washed with 200 ml. sterile water before use.

The sea water used was sampled at Woods Hole and at Nahant, Mass. When not used immediately it was stored at 3°. For pure culture studies artificial sea water (Lyman & Fleming, 1940) was used.

Marine bacteria were isolated on modified 2216E agar medium (Oppenheimer & ZoBell, 1952) and were grown in liquid 2216E medium in 250 ml. Erlenmeyer flasks at 23°.

Degradation studies of *Pythium debaryanum* in both natural sea water and by pure cultures were carried out in 250 ml. Erlenmeyer flasks at 25° on a new Brunswick (Model G10) gyratory shaker. Mycelial mat weight was determined following collection of the fungus by filtration and drying at 110°, always in triplicate.

Cell-free filtrates were obtained after degradation of the mat in natural sea water by centrifuging for 10 min. at 7000 *g* and then aseptically filtering through 0·45 μ Millipore filters.

Some of the sea-water filtrate was concentrated by reducing the volume in a reverse osmosis ultrafiltration cell. Molecules with a molecular weight greater than 10,000 are retained by the UM-1 ultrafiltration membrane. The apparatus (Diaflo Model 50 cell) and membranes were obtained from the Amicon Corp., Cambridge, Mass. Enzymic activity of the filtrates was tested against washed Pythium mycelium by assaying for release of reducing sugars by the anthrone method (Dische, 1955). Enrichment cultures were employed using Pythium cell walls prepared in a modified French press (Raper & Hyatt, 1963).

RESULTS

Effect of the marine microflora on Pythium mycelium

In order to study the effect of the native marine microflora on Pythium mycelium, mycelial mats were added to sterile and non-sterile sea water. The mats remained intact in the sterile sea water, but were slowly degraded in static culture at 23° in fresh non-sterile sea water. Total degradation usually occurred 26 days after inoculation of the fungus.

The development of a specific antagonistic marine microflora was demonstrated by adding washed Pythium mycelial mats to fresh natural sea water, to autoclaved sea water, and to Millipore-filtered (0·22 μ) sea water. The mycelia shaken in natural sea water completely disintegrated in 12 days, whereas in autoclaved and filtered sea water the mycelium was unaffected (Fig. 1).

When a second Pythium inoculum was placed in the flasks following degradation and subsequent removal of the remaining first mycelial mat, the second mat degraded, but much more rapidly, the time of degradation being 6 days (Fig. 1).

These data indicate either that the first Pythium inoculum stimulated the development of an antagonistic microflora which rapidly degraded the second inoculum of the fungus, or that an inducible enzyme system was stimulated.

Effect of pH, nutrients and dilution on the degradation of mycelium

Mycelial mats were placed in filter-sterilized sea water adjusted to pH values of 4, 5, 8 and 9. A loopful of inoculum from a Pythium culture being actively degraded in sea water was added to each flask. After 7 days the mats at pH 5, 8 and 9 showed signs of degradation, with the most extensive degradation occurring at pH 8. There was no degradation at pH 4 and the mats remained intact. It was observed that when 10 p.p.m. of 100 p.p.m. yeast extract was added to sea water along with a Pythium mat, the degradation process was accelerated. The process was accelerated even more when

yeast extract was added to the sea water 2 days before addition of the fungus, indicating that a stimulation of the native marine microbial population accelerated fungal lysis.

An indirect effect of bacterial population size was shown by dilution of the natural sea water. Sea water was sterilized by Millipore-filtration (0·22 μ). The undiluted samples contained 100 ml. natural sea water. The 1/10 dilution contained 90 ml. filter-sterilized sea water and 10 ml. natural sea water. The 1/100 dilution contained 99 ml. filter-sterilized sea water and 1·0 ml. natural sea water. The time required for the degradation of fungal mats added to the flasks was followed by measuring mycelial mat weight. The mats in undiluted sea water displayed visible degradation after 4 days

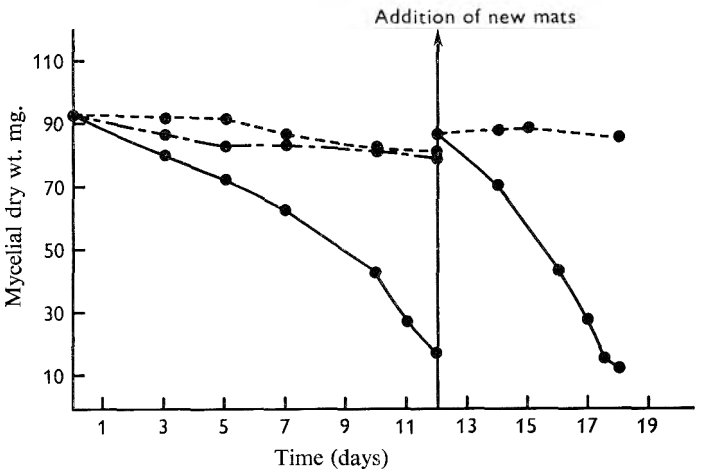

Fig. 1. Effect of repeated additions of mycelial mats on lysis of *Pythium debaryanum* mycelium in natural sea water. ——, Natural sea water, – – –, autoclaved sea water; —·—, Millipore-filtered (0·22 μ sea water).

and were totally degraded after 8 days; the mats in 1/10 dilution began to lyse after 7 days and were totally degraded after 12 days; the mats in the 1/100 dilution began to lyse after 9 days and were totally degraded after 17 days. These data provide further evidence that the degradation of Pythium is directly proportional to the size of the native marine microbial population.

Tests for enzymic activity

In subsequent experiments the ability of cell-free filtrates of sea water to degrade Pythium mycelium enzymically was tested. We used sea water which had been exposed to and had degraded two consecutive Pythium inocula, so as to obtain maximal activity. When the second inoculum was completely degraded the sea water was centrifuged for 20 min. at 7000 *g* and filtered aseptically through 0·45 μ Millipore filters. Some of the sea water filtrate was concentrated by reducing the volume in a reverse osmosis ultrafiltration cell. Molecules with a molecular weight greater than 10,000 were retained by the ultrafiltration membrane used in this study. Concentration of these molecules was achieved within approximately 1 hr in a single step using this method.

The concentrated and unconcentrated cell-free filtrates were tested for enzymic

activity against intact washed Pythium mycelium. The results obtained (Fig. 2) show that reducing sugars, measured as μg. glucose/ml., were released within 1 hr after addition of the mycelium to unconcentrated filtrate. The activity was substantially increased in the concentrated filtrate. The system was inactivated by heating the filtrate to 100° for 15 min. These data indicate the presence of extracellular lytic enzymes produced by marine micro-organisms which suppressed the non-marine fungus.

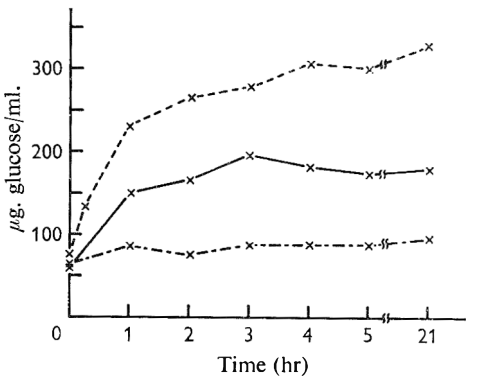

Fig. 2

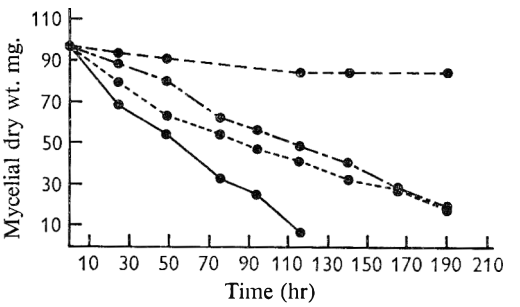

Fig. 3

Fig. 2. Release of reducing sugars during lysis of *Pythium debaryanum* mycelium by filtered sea water. The sea water was subjected to repeated inoculation with Pythium before filtration. ——, Unconcentrated filtrate; – – –, concentrated (11·5 ×) filtrate; —·—, boiled filtrate.

Fig. 3. Relationship between the numbers of lytic bacteria present and the rate of lysis of *Pythium debaryanum*. – – –, Autoclaved sea water; – — –, sea water and 10^4 bacteria/ml. added; - - -, sea water and 10^5 bacteria/ml. added; ——, sea water and 10^6 bacteria/ml. added.

Pure culture studies

Isolations of marine bacteria were made from sea water to 2216E media following degradation of a mycelial mat. A bacterium, tentatively identified as an *Agarbacterium* sp. which would degrade Pythium in pure culture, was isolated from sea water enriched with Pythium cell walls. This bacterium yielded results similar to those observed in natural sea water. However, the degradation time was more rapid in pure culture. Fig. 3 shows data indicating that when the cell concentration of *Agarbacterium* sp. added to artificial sea water was 10^4/ml., the fungal mat was degraded in 190 hr. When the concentration was 10^6/ml., degradation occurred in 116 hr. A higher level of antifungal activity was detected following repeated inoculation of the fungus. When a fungal mat was added to artificial sea water inoculated with 10^6/ml. *Agarbacterium* sp., the mat was degraded in 115 hr, whereas the second inoculum of the fungus was degraded by the bacteria in 75 hr (Fig. 4). Many spheroplasts of the fungus were observed under phase-contrast microscopy during the course of degradation, suggesting that lysis of the fungal cell walls was occurring.

Comparison of lysis of a marine-isolated Pythium with Pythium debaryanum

Mycelial mats of *Pythium debaryanum* grown in potato dextrose broth were washed and added to flasks containing 100 ml. autoclaved sea water and to flasks containing

100 ml. of natural sea water. The same procedure was followed for a marine isolate of Pythium. The marine species was grown in potato dextrose broth prepared with 75 % sea water. Mycelial mat weights were determined, in triplicate, during degradation. The results presented in Fig. 5 show that in the time period tested the mycelial stage of the marine isolate was as susceptible to degradation by the marine microflora as *Pythium debaryanum*. This may imply salt adaptation on the part of the marine isolate with no or very slight changes in its cell-wall structure, thus giving it no competitive advantage over the freshwater strain.

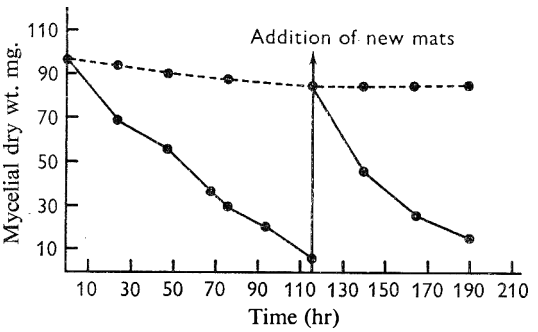

Fig. 4

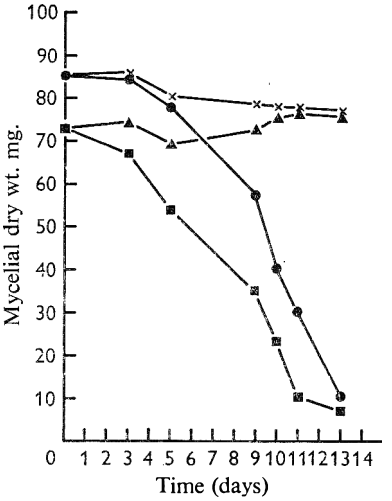

Fig. 5

Fig. 4. The effect of repeated additions of mycelium on degradation of *Pythium debaryanum* by a lytic bacterium isolated from the sea. – – –, Autoclaved sea water and 10^6 bacteria/ml. added.

Fig. 5. A comparison of the rates of lysis in natural sea water of a freshwater strain and a marine-isolated Pythium strain. ▲—▲, Salt water Pythium control; ■—■, salt water Pythium test; × — ×, freshwater Pythium control; ●—●, freshwater Pythium test.

The effect of salt adaptation on the susceptibility to lysis of the non-marine Pythium was tested by first adapting *Pythium debaryanum* to grow in potato dextrose broth prepared in 80 % artificial sea water. This was achieved by a series of serial transfers to 10, 20, 40, 60, 70 and 80 % artificial sea water. The fungus was then tested for resistance to degradation. It was observed that the salt-adapted *Pythium debaryanum* was degraded as rapidly as the non-adapted *P. debaryanum* both in natural sea water and in artificial sea water with 10^6/ml. *Agarbacterium* sp. added.

Degradation studies of other Oomycetes

Five isolates of closely related Oomycetes were tested for resistance to degradation in natural sea water and also to degradation by the Pythium-degrading bacterium. The isolates were *Apodachlya brachynema*, *Saprolegnia diclina*, *Achlya caroliniania*, *Isoachlya luxuriana* and *Thraustotheca clavata*. Mycelial mats of these fungi were grown in potato dextrose broth, collected, washed, and added to 100 ml. natural sea water and to 100 ml. autoclaved sea water in 250 ml. Erlenmeyer flasks. All of the mats in natural sea water turned yellowish and were entirely degraded in no more than 8 days in shake culture. *Apodachlya brachynema* was degraded in 8 days; *S. diclina* in 5 days,

A. caroliniania in 7 days, *I. luxuriana* in 6 days, and *T. clavata* in 5 days. The mats in sterile sea water remained white, perfectly intact and viable.

These five fungi were then tested with the *Agarbacterium* sp. previously found capable of decomposing Pythium in sterile sea water. Washed mycelial mats were added to sterile sea water and an inoculum of 10^6 bacteria/ml. was added. All five fungi were susceptible to lysis by this bacterium in no longer than 5 days. *Traustotheca clavata*, *Saprolognia diclina* and *Apodachlya brachynema* were completely degraded in 3 days; *Isoachlya luxuriana* in 4 days; and *Achlya caroliniania* in 5 days.

Thus it appears that there is a specific microflora which attacks the mycelial stage of these fungi in sea water. The same bacterial strain isolated from sea water was active against all of the fungi tested. These results indicate a close similarity in cell-wall structures of these fungi.

DISCUSSION

Large numbers of fungi have been isolated from the sea (Johnson & Sparrow, 1961). We do not know if all these fungi grow actively in the sea, or if the isolates simply reflect survival of these fungi in the form of spores. We have observed that when *Escherichia coli* is added to sea water, an antagonistic marine microflora develops which kills that bacterium (Mitchell, Yankofsky & Jannasch, 1967). In the current investigation, we present evidence that an antagonistic marine microflora also develops in response to the addition to sea water of the mycelial form of several Oomycetes.

The observation that the population size of the marine microflora affects the rate of kill of the fungus provides further evidence indicating the implication of a group of native marine micro-organisms in the eradication of *Pythium debaryanum* in the sea. The data showing that the rate of lysis of Pythium mycelium increases with each subsequent addition of the fungus to the same sample of sea water suggest the enrichment of a specific antagonistic group of micro-organisms or the increase in adaptive enzyme capacity or both.

We have reported previously the enzymic lysis of *Pythium debaryanum* in soil (Mitchell & Hurwitz, 1965). It was not surprising, therefore, to find that culture filtrates of sea water contained enzymes capable of lysing Pythium following exposure of the sea water to mycelium of that fungus. We did not expect, however, that such large quantities of enzyme would be active in the sea water. Both enzymes (Mitchell & Nevo, 1965) and Bdellovibrios (Mitchell *et al.* 1967) have been associated with the killing of *Escherichia coli* in sea water. Apparently, enzymic lysis of the cell walls is solely responsible for the decline of Pythium.

A similar susceptibility to lysis was detected when other non-marine Oomycetes were added to sea water. The observation that a marine isolated Pythium strain was equally susceptible to lysis suggests that fungi isolated from marine estuaries may not in fact, be ecologically restricted to the marine environment. Resistance to lysis by a marine microflora may be a useful tool in the characterization of a true marine mycoflora.

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Fine Structure of *Thiobacillus novellus*

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SUMMARY

The study of ultrathin sections of *Thiobacillus novellus* has shown that it has a structure similar to that of *T. thiooxidans* and other Gram-negative bacteria. The cell wall showed a multilayered structure and varied in thickness between 250 and 350 Å. The cytoplasmic membrane and 70 Å thick. On certain media a capsule was formed. The cytoplasm contained ribosomes and polymetaphosphate granules. The structure of the nuclear material was similar to that observed in other bacteria.

INTRODUCTION

Thiobacillus novellus Starkey 1935 represents an exception among species of the genus *Thiobacillus*. While the other species of the genus are both motile and obligate autotrophic *T. novellus* is non-motile and facultative autotrophic.

Sijderius (1946) was of the opinion that *Thiobacillus novellus* was identical with *Micrococcus denitrificans*. The mentioned species, however, differ both in their ultrastructure (Kocur, Martinec & Mazanec, 1968) and in their % GC values in the deoxyribonucleic acid (Boháček, Kocur & Martinec, 1965). Therefore they cannot be considered as identical.

The purpose of this paper was to give a description of the fine structure of *Thiobacillus novellus* and compare it with the structures of similar organisms already described by other authors.

METHODS

Organism. The strain *Thiobacillus novellus* CCM 1077 (ATCC 8093) used in the present study came from the Czechoslovak Collection of Microorganisms, J. E. Purkyně University, Brno. The cells were grown on two different media: on the medium for autotrophic growth of *T. novellus* recommended by Santer, Boyer & Santer (1959) and on the medium of Chang & Morris (1962).

Electron microscopy. After 24 hr cultivation at 30°, the cells were flushed with saline, washed three times and centrifuged for 5 min. After 2 hr fixation with 1% solution of OsO_4 in phosphate buffer at pH 7·3 according to Millonig (1962), the cells were flushed with appropriate buffer and refixed with 10% neutral formol at 4° for 1 hr. After having been washed with saline the cells were centrifuged and embedded in 1·3% agar and dehydrated with alcohol. The dehydrated blocks were embedded in Epon 812 according to the prescription of Luft (1961).

Ultrathin sections made with an ultramicrotome, type Tesla Bs478, were stained for 20 min with a 2% solution of uranylacetate (pH 5) and for 10 min with lead citrate according to Reynolds (1963). The sections were studied and photographed in an electron microscope, type Tesla Bs413 A.

RESULTS

In the same preparation of *Thiobacillus novellus* two types of cells were observed: short rods, measuring 0·8 to 1 × 1·7 μ and longer ones, the size of which was 1 × 3·5 μ. As can be seen from Pl. 1, fig. 1, 2 the cell wall (CW) has a multilayered structure characteristic of Gram-negative bacteria and its total thickness ranges from 250 to 350 Å (see Pl. 3, fig. 5, 6).

On certain cultivating media a capsule was formed. When cultivated in the medium of Chang & Morris (1962) the cell wall of *Thiobacillus novellus* was covered with a mucous layer the thickness of which ranged from 700 to 1000 Å (Pl. 3, fig. 5 and 6). But when cultivated in the medium of Santer *et al.* (1959) it did not form a capsule (Pl. 1, fig. 1, 2; Pl. 2, fig. 3, 4; Pl. 3, fig. 7, 8).

The cytoplasmic membrane is a typical unit membrane 80 Å in width and corresponds by its structure and thickness to other Gram-negative bacteria. It can best be seen in Pl. 2, fig. 4. The cytoplasm is of granular structure and contains: (i) electron-dense particles—ribosomes (R); (ii) inclusions (P). No mesosomes were observed.

There occur frequently light areas in *Thiobacillus novellus*, looking like vacuoles situated either in the end (Pl. 1, fig. 1, 2) or in the central part of the organism (Pl. 3, fig. 5). These areas are separated from the cytoplasm by a membrane.

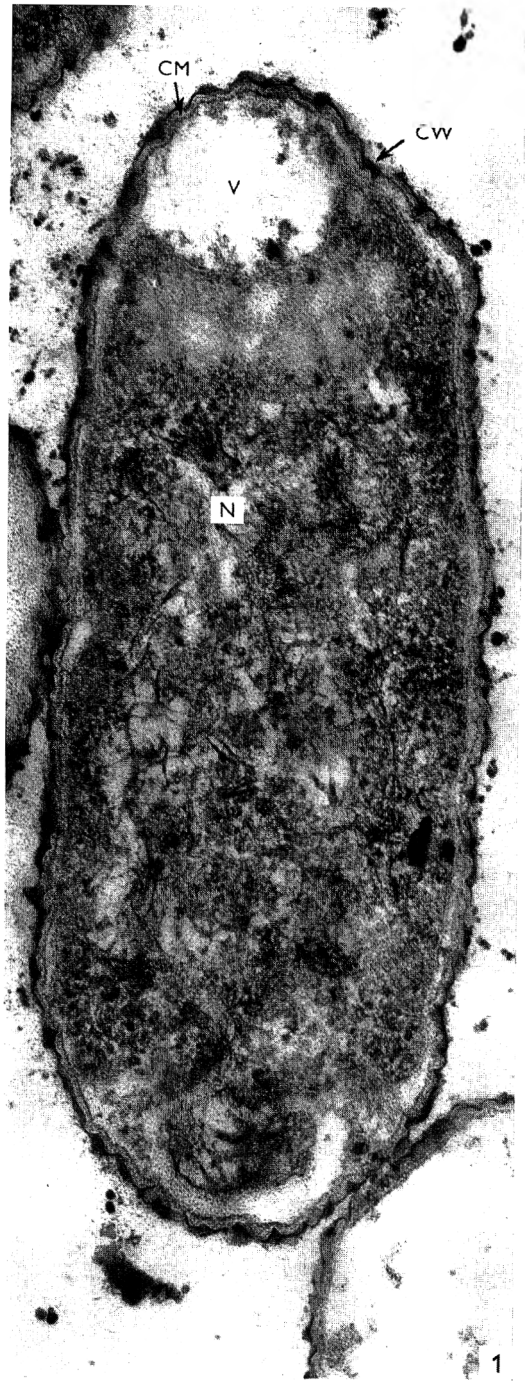
The nuclear material of *Thiobacillus novellus* has a typical filamentous structure of highly varied course. It is clearly distinguishable from the granular cytoplasm by its light colour.

Division in *Thiobacillus novellus* proceeds in the same way as in other Gram-negative bacteria (Conti & Gettner, 1962; Mahoney & Edwards, 1966). Different stages of division are shown in Pl. 3, fig. 7, 8. Plate 3, fig. 7, shows the early stage of division—the cytoplasmic membrane and the cell wall begin to invaginate into cytoplasm. As can be seen from Pl. 3, fig. 8, each daughter cell has its own continuous cytoplasmic membrane (see arrows), while the cell wall is common to both cells.

DISCUSSION

The results obtained in the present work showed that the cell-wall structure of *Thiobacillus novellus* is similar to that of other Gram-negative bacteria (Mahoney & Edwards, 1966; Remsen & Lundgren, 1966). At present, the fine structure of only a single species of the genus *Thiobacillus*, *T. thiooxidans*, is known (Mahoney & Edwards, 1966). The comparison of its electron micrographs with those of *T. novellus* showed that these organisms have a similar cell structure. No differences in the cell-wall structure explaining the different physiology of the facultative autotrophic *T. novellus* and the strict autotrophic *T. thiooxidans* were observed. *T. novellus* differs from *T. thiooxidans* only in its greater size, in non-flagellated cells which can be encapsulated under certain conditions.

The internal structure of both organisms is similar. We found in *Thiobacillus novellus* large electron-dense bodies similar to those observed by Mahoney & Edwards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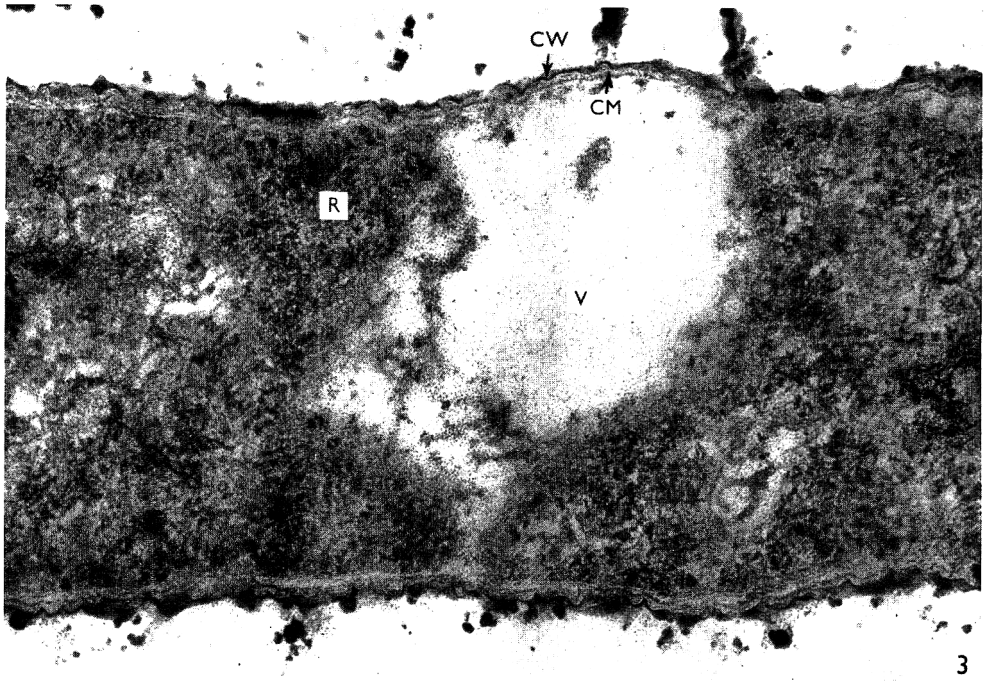

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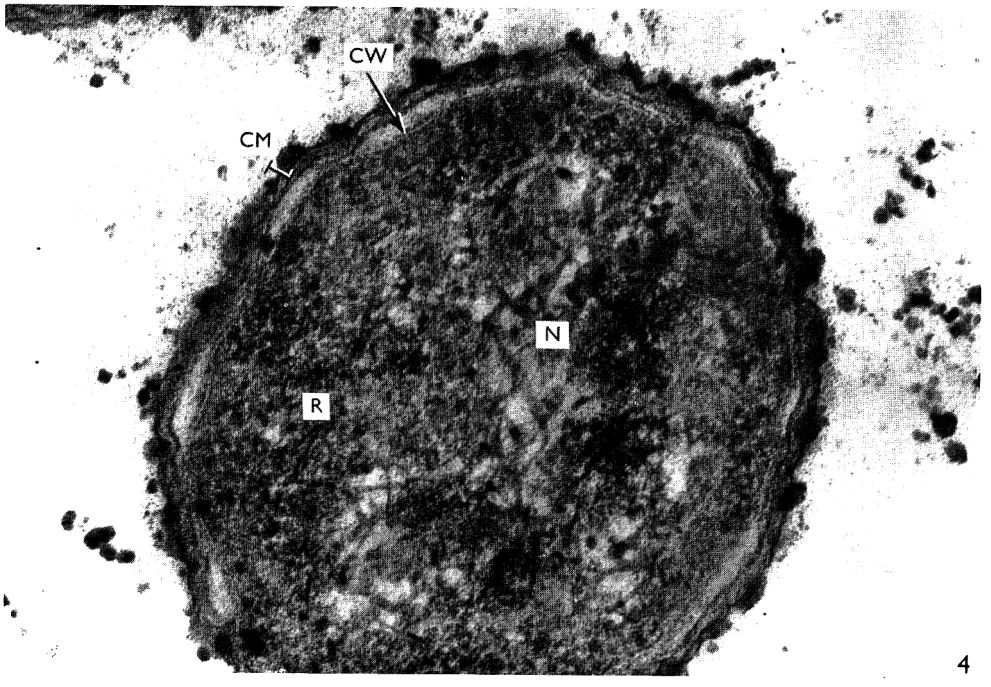

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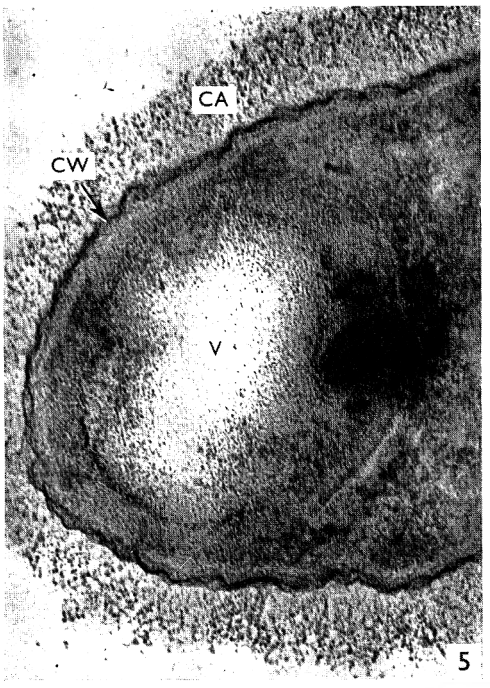
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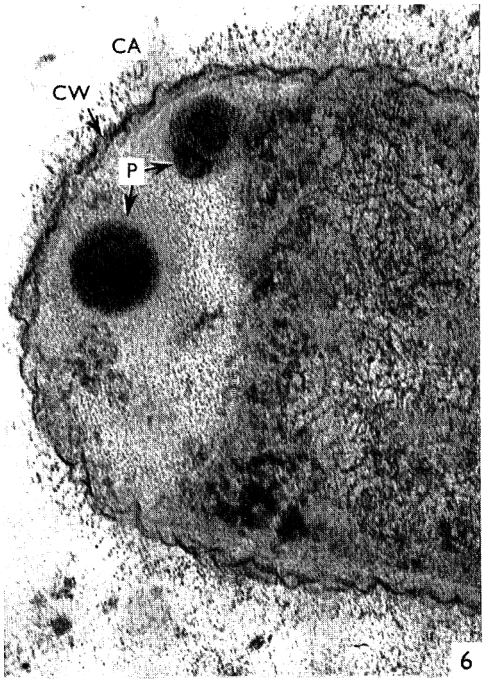
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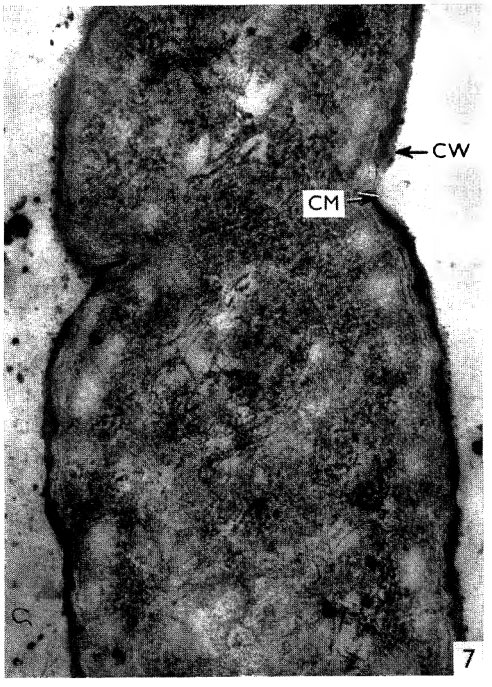
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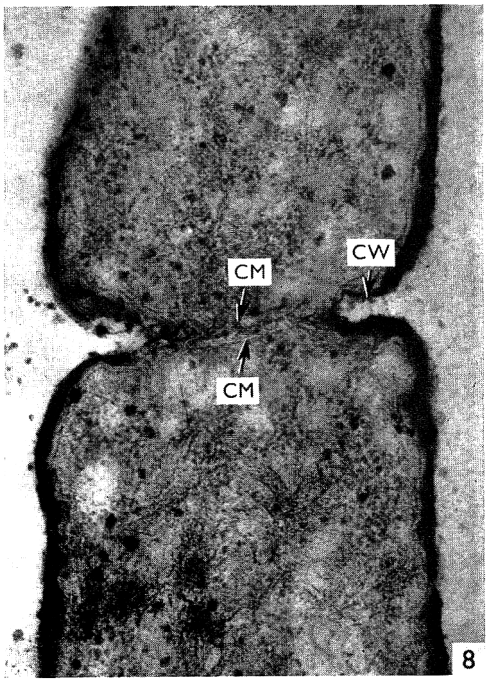
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8

(1966) in *T. thiooxidans*. These osmiophilic bodies are most probably composed of polymetaphosphate. As for the light areas occurring in *T. novellus*, these are considered to be artifacts produced by plasmolysis as they are situated between the cell wall and the cytoplasmic membrane. It is known that plasmolysis can occur in various stages of preparation of the blocks for ultrathin sections.

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EXPLANATION OF PLATES

PLATE 1

Fig. 1, 2. Longitudinal section showing cell wall (CW), cytoplasmic membrane (CM), nuclear material (N), vacuole-like area (V). Fig. 1, ×95000; fig. 2, ×75000.

PLATE 2

Fig. 3, 4. Longitudinal and cross-sections showing cell wall (CW), cytoplasmic membrane (CM), nuclear material (N) and ribosomes (R). Fig. 3, ×75,000; fig. 4, ×125000.

PLATE 3

Fig. 5, 6. Portion of cells showing capsules (CA), cell wall (CW), cytoplasmic membrane (CM), polymetaphosphate granule (P) and vacuole-like area (V). Fig. 5, ×85,000; fig. 6 ×85,000.
Fig. 7, 8. Portion of cells showing various stages of cell division. Cell wall (CW), cytoplasmic membrane (CM). Fig. 7, ×75,000; fig. 8, ×75,000.

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Salt Dependency of the Bacterial Flora of Marine Fish

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SUMMARY

The bacterial flora on the skin of plaice (*Kareius bicoloratus*) caught in coastal regions of Japan was composed of bacterial groups heterogenous to the concentration of salt in their growth behaviour. Each bacterial group had a different minimum and/or maximum salt concentration for growth, and different groups were selected by media with different salt concentration. In the media based on two complex salt solutions, including an artificial sea water, the maximum bacterial counts were obtained at concentrations corresponding to about 25% dilution of sea water. During the storage of fish at 24° and 2°, bacteria which required higher salt concentrations for growth increased faster than that of non-halophilic bacteria. The microflora of the digestive tract of the fish was composed almost exclusively of halophilic vibrios.

INTRODUCTION

A previous survey on the sea water from coastal areas of Japan (Simidu & Aiso, 1962) showed that an appreciable number of sea water bacteria required at least 0·2% sodium chloride for their growth. Most of these bacteria retained their halophilic nature after serial subcultures. These results suggested that a large proportion of the bacteria of fish from the same environment might also be halophilic in nature. If this were the case, the halophilic nature of fish bacteria might be of some significance in the spoilage of fish. Although many workers presuppose the halophilic nature of fish bacteria, there is little information on the actual distribution of halophilic bacteria in the microflora of marine fish; and their possible role before and after the death of the fish is still obscure. In the present report, the growth behaviour of the bacterial population as well as of individual isolates from marine fish were studied at different salt concentrations.

METHODS

The experiments were done during the months October to May.

Plaice, 'Ishigarei' (*Kareius bicoloratus*), weighing 120–680 g., were bought at Funabashi Fish Market, Chiba Prefecture. The fish had been sent alive to the market without refrigeration. Although 4 to 5 hr had elapsed from the time of catching, the fish were in most cases still alive at the beginning of the experiment. Skin of the middle dorsal part of the fish (6·0 or 9·0 cm.^{2} in area) with or without underlying muscle, was excised aseptically from the fish. The skin sample was homogenized with 50–100 ml. of a salt solution: 0·85% NaCl in the experiments to see the effect of NaCl solution and an artificial sea water, and a mixed solution consisting of 0·8% NaCl

and 1·0% KCl in the experiments with solution C. From serial dilutions, 0·5 ml. of the homogenate was placed in triplicate on plates by using a mechanical pipette (Yanagita & Maruyama, 1953) to ensure a fast and accurate procedure. Agar medium (20 ml.) was poured into the plate; plates were incubated at 23 to 25° for 4 to 7 days.

Three different mixtures of medium and salt solution were used. In the first series of experiments, 1·0% Polypepton (Daigo Eiyo Kagaku Co., Osaka), 0·5% meat extract (Kyokuto Seiyaku Kogyo Co., Tokyo) and 1·2% agar were dissolved in solutions of sodium chloride of various concentrations. In the second series, the same constituents were dissolved in an artificial sea water (*Kaiyo no Jiten*, 1960, p. 305, Table 1) at various dilutions. For the third series of experiments, 1·0% Polypepton, 0·3% Lab-Lemco (Oxoid), 0·3% yeast extract (Difco) and 1·2% agar were dissolved into a complex salt solution (solution C, Table 1) of various dilution. After counting the colonies, about 30–40 colonies were transferred from a randomly enclosed area of each plate of different salt concentration into the broth media of the same composition as those used for the counting.

The growth dependency of the isolated strains on salt concentration was examined by streaking on plates of agar medium containing 1·0% Polypepton and various concentrations of sodium chloride. The Polypepton was reported to contain 2·2% sodium, 0·5% calcium and a trace amount of potassium.

Table 1. *Composition of basal salt solution*

	Artificial sea-water		Solution C	
	M	g./l.	M	g./l.
NaCl	0·403	23·5	0·250	14·6
KCl	0·089	6·6	0·250	18·6
$Na_2SO_4.10H_2O$	0·028	8·9	—	—
$MgCl_2.6H_2O$	0·049	10·2	—	—
$MgSO_4.7H_2O$	—	—	0·025	6·2
$CaCl_2.2H_2O$	0·010	1·5	0·010	1·5

RESULTS

Viable counts and the concentration of sodium chloride

Plate counts were made on nutrient agar medium containing different amounts of sodium chloride with skin with underlying muscle from a plaice. The remaining part of the same fish was stored for 24 hr at 24° and plate counts then made on the stored fish. Plates were incubated at 24°, and colonies counted after 4 days. Results from a representative experiment are illustrated in Fig. 1. Repeated experiments with plaice and with sardine (*Sardinops melanosticta*) gave similar relationships of viable count with increasing salt concentration. In all cases the counts showed two peaks at higher salt concentrations, although the position and relative height of the peaks differed in different experiments. The increasing tendency for the bacterial flora to become more halophilic during storage of fish was confirmed in repeated experiments.

Heterogeneity of the isolated strains to salt concentration

The growth of the isolates from the experiment illustrated in Fig. 1 was examined on peptone agar containing NaCl (%, w/v): 0, 0·2, 0·5, 1, 2, 3, 5, 7, 10, 15. Before

testing the growth in these NaCl concentrations the strains were grown in nutrient broth containing the concentration of sodium chloride as was in the media used for the initial isolation.

Table 2. *Relation between the range of salt concentration in which the isolates could grow and NaCl concentration of the media used for initial isolation*

Isolates from media containing 1·0, 2·0 and 3·0% NaCl were inoculated on 1% peptone agar containing different NaCl concentrations. Growth was determined after 5 days of incubation at 25°.

Range of NaCl concn. (%)	Concn. of NaCl in media used for the initial isolation from plaice (%)					
	1·0	2·0	3·0	1·0	2·0	3·0
	Isolates from fresh plaice			Isolates from plaice stored 1 day at 24°		
	No. of strains grown in the range of NaCl concn. given in the first column					
0–10	0	0	3	0	0	0
0–7	1	0	4	2	0	0
0–5	3	1	2	5	1	4
0–3	4	3	3	2	1	1
0–2	6	6	0	0	2	0
0–1	3	4	1	0	0	0
0·2–7	0	0	0	1	1	0
0·2–5	0	0	0	1	0	1
0·2–3	0	0	0	1	0	0
0·2–2	0	1	0	0	0	0
0·2–1	3	0	0	0	0	0
0·5–7	0	0	0	0	0	2
0·5–5	0	0	0	1	0	3
0·5–3	0	1	2	7	5	7
0·5–2	0	0	0	0	2	0
0·5–1	1	0	0	0	0	0
1–7	0	0	0	1	0	0
1–5	0	1	0	0	1	0
1–3	3	2	6	5	10	4
1–2	0	0	0	0	5	0
1	0	1	0	0	0	0
2–5	0	0	1	0	0	0
2–3	0	7	2	0	1	5
2	0	0	1	0	0	0
3	0	0	1	0	0	1
No growth	2	2	1	2	0	1
Total	26	29	27	28	29	29

The results (Table 2) showed that the bacterial flora of fish consisted of bacteria with different minimum and maximum NaCl concentrations for growth and that different bacterial groups were selected according to the concentration of NaCl of the media. After storage for a day at 24°, the numbers of bacteria which could not grow at NaCl concentrations of lower than 0·2% had increased remarkably.

The generic composition of the isolates determined according to the scheme of Shewan, Hobbs & Hodgkiss (1960) is shown in Table 3. Although the numbers of strains examined were not sufficient to draw decisive conclusions, the results seemed to show heterogeneity among the microfloras obtained from the isolation media of

different NaCl concentrations. Gram-positive bacteria tended to decrease in numbers with increasing NaCl concentration, pseudomonads and vibrios seemed to increase at higher NaCl concentrations.

Table 3. *Generic composition of bacteria isolated with media of different NaCl concentrations*

	Concn. of NaCl in media used for the isolation (%)							
	0	1·0	2·0	3·0	0	1·0	2·0	3·0
Bacterial genus	Isolates from fresh plaice				Isolates from plaice stored 1 day at 24°			
Pseudomonas	1	4	8	4	3	16	23	21
Vibrio	0	0	6	7	7	5	2	5
Achromobacter	13	7	1	6	13	4	0	0
Flavobacterium	7	5	8	5	2	1	0	0
Corynebacterium	3	6	3	1	3	0	2	1
Micrococcus	4	0	1	1	1	1	2	1
Bacillus	2	0	0	3	0	0	0	0
Moulds and yeast	0	1	1	0	0	0	0	0
Not identified	0	3	1	0	0	1	0	1
Total	30	26	29	27	29	28	29	29

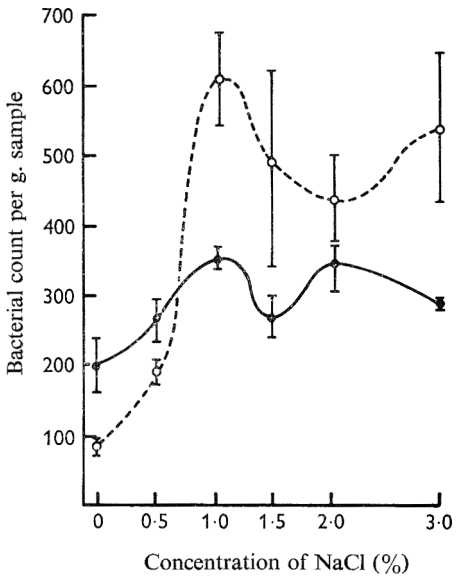

Fig. 1

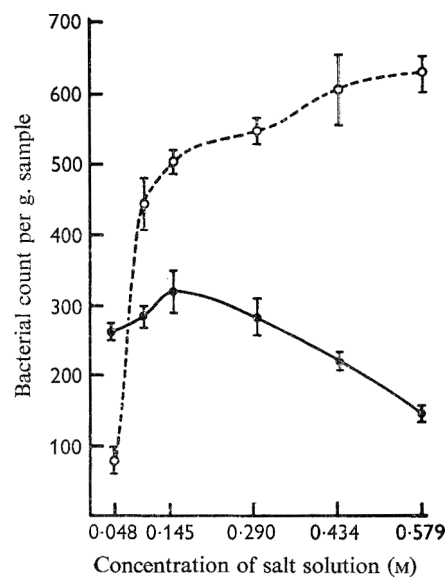

Fig. 2

Fig. 1. Relation between viable counts and salt concentration of medium. Results with skin with muscle from: ●——●, fresh plaice (count $\times 10^{-3}$); ○- - -○, plaice stored 24 h at 24°, (count $\times 10^{-5}$). Vertical lines show standard deviations about means of triplicate plates.

Fig. 2. Relation between viable count and concentration of artificial sea-water in medium. Results with skin + muscle from: ●——●, fresh plaice (count $\times 10^{-3}$); ○- - - - -○, plaice stored 24 hr at 24° (count $\times 10^{-6}$).

Experiments with complex salt solutions

The use of artificial sea water or other complex salt solutions as a basal solution in the isolation medium was expected to eliminate the inhibitory effect of sodium ion on non-halophilic bacteria and to give different patterns in relation to viable count and salt concentration. Figure 2 shows the results with an artificial sea water, the composition of which is given in Table 1. The plate counts were made of skin with underlying muscle from a plaice, and colonies counted after the incubation for 4 days at 25°.

During experiments to obtain a medium which would give the highest viable count from a fish sample, a salt solution (solution C, Table 1) was developed (Simidu & Hasuo, 1968). The relation between the viable count of the fish sample and the concentration of this solution was examined. The composition of the basal medium was (%, w/v): 1·0, Polypepton; 0·3, Lab-Lemco; 0·3, yeast extract; 1·2, agar. The constituents were dissolved in solution C at different dilutions. The media were adjusted to pH 7·5.

Table 4. *Relation between the range of salt concentration in which the isolates could grow and the concentration of salt solution (solution C, Table 1) in the media used for the initial isolation*

Isolates were inoculated on 1% peptone agar containing different NaCl concentrations. Growth was determined after 5 days of inoculation at 25°.

Range of NaCl Concn. (%)	Concentration of salt solution C in media used for the initial isolation (M)		
	0·134	0·268	0·401
	No. of strains grown in the range of NaCl given in the first column		
0–10	3	1	1
0–7	3	3	4
0–5	5	7	10
0–3	10	10	9
0–1	6	5	1
0·3–7	9	2	2
0·3–5	2	2	3
0·3–3	2	2	0
0·3–1	2	1	0
1–7	0	2	6
1–5	0	1	3
1–3	0	1	0
3	0	0	1
Total	43	37	40

Skin with underlying muscle was removed with a sterile knife from the back middle part of plaice weighing 675 g. and cut into three parts. One of these was used immediately for the experiment, and the other two parts were kept in large sterile dishes and stored at 2° and 24°, respectively, to await further experiments. To protect these samples from drying, a small sterile dish of water was laid beside the cut skin. Skin, 6·0 cm.^{2} in area, was excised aseptically from each part, and homogenized with a diluent composed of 0·8% NaCl + 1·0% KCl. Plate counts were made of serial

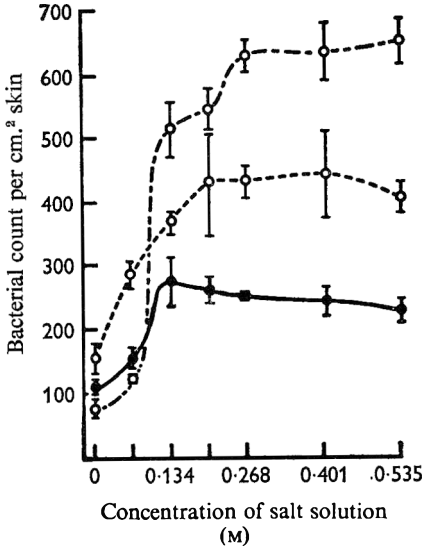

Fig. 3

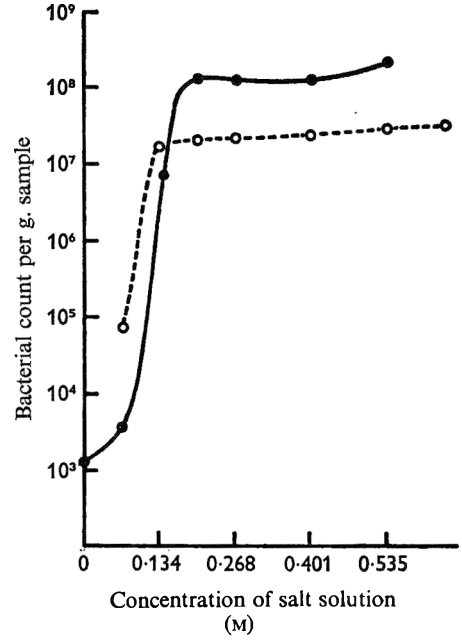

Fig. 4

Fig. 3. Relation between viable count and concentration of a complex salt solution (solution C) in medium. Results with skin of: ●——●, fresh plaice (count × 10^{-3}); O– — –O, plaice stored 24 hr at 24° (count × 10^{-7}); O - - - - O, plaice stored 5 days at 2° (count × 10^{-6}).

Fig. 4. Relation between viable counts and concentration of a complex salt solution (solution C) in medium. ●——●, 1st experiment, O - - - - - O, 2nd experiment.

Table 5. *Generic composition of bacterial flora from the digestive tract of plaice determined by the scheme of Shewan et al. (1960)*

Bacterial genus	Concentration of salt solution (solution C, Table 1) in media used for the initial isolation (M)	
	0·134	0·535
	No. of strains	
Vibrio	38	37
Pseudomonas	2	3
Total	40	40

dilutions and the resulting colonies counted after 4 days at 24°. The results obtained are given in Fig. 3.

The range of salt concentration in which the isolated bacteria from fresh fish grew was determined with the peptone agar of different NaCl concentrations (%, w/v), 0, 0·3, 1, 3, 5, 7, 10, 15. Care was taken to eliminate the effect of different NaCl concentrations during precultivation: before streaking on peptone agar, all the strains tested were transferred twice into the medium composed of 1·0% Polypepton,

0·3 % Lab-Lemco, 0·3 % yeast extract and half strength C solution. A few isolates which failed to grow at this salt concentration were precultured in medium of either full strength or $\frac{1}{5}$ strength of the salt solution. The results after 5 days of incubation at 24° are summarized in Table 4. Although there was no appreciable difference among the total numbers of the bacteria initially grown at the three different given concentrations of salts (Fig. 3), the isolated bacteria gave different patterns of growth range according to the salt concentration at the initial isolation.

Along with the experiments with the skin of fish, the relation between growth and salt concentration was examined with the microflora of the digestive tract of the fish. The medium used was the Lab-Lemco + yeast extract agar dissolved in the salt solution C at different dilutions. Figure 4 shows the results obtained from two experiments, the first with a smaller (216 g.) and the second with a bigger (440 g.) fish. The generic composition of the isolates from the second experiment is given in Table 5.

DISCUSSION

ZoBell (1941) studied the salt dependency of the bacterial flora from various sources and showed that maximum viable counts were obtained at the salt concentration corresponding to sea water for the flora of sea water, and at 10–25 % dilution of sea water for the flora of sea mud from terrestrially polluted bays. Our results with an artificial sea water showed that the highest counts were obtained at 25 % dilution of sea water when fresh marine fish were examined. In the experiment with stored fish, the highest counts were obtained at higher salt concentrations A similar relation between salt concentration and viable counts was observed in the experiments with another complex salt solution in which the ionic ratio of sodium to potassium was 1 : 1.

The faster growth of halophilic bacteria at lower as well as higher temperatures is a factor which should be taken into account on the cold storage of fish. Washing fish with tap water or direct contact of fish with ice might eliminate, as is believed by some Japanese workers, the numbers of halophilic bacteria, and might alter the rate and pattern of the spoilage of the fish.

A striking feature observed in the present work was the strong halophilic nature of the bacterial flora from the digestive tract of marine fish. Liston (1957) reported the occurrence of the 'Gut group vibrios' in the gut contents of fish from the North Sea. Whereas the ratio of the vibrios in Liston's experiments was 35–74 % of the total gut flora, the present study showed the gut flora of plaice caught in coastal areas of Japan to be composed almost exclusively of vibrios, and suggests that these vibrios constitute the indigenous microflora of fish intestine.

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An Improved Medium for the Isolation of Bacteria from Marine Fish

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SUMMARY

A medium for the isolation of heterotrophic aerobic bacteria from marine fish has been developed. The medium contains inorganic ions with a total concentration equal to about half that of sea water. The ionic ratio of sodium and potassium in the medium is nearly 1:1, and the concentration of the organic nutrients is higher than in media that have been generally used for the isolation of bacteria from sea water.

INTRODUCTION

One of the determining factors in the investigation of the bacterial flora of marine fish is the composition of the media used for the isolation of the bacteria. After the study of ZoBell (1941) on the cultural requirements of bacteria from sea water, many investigators have used the medium developed by him, often with various modifications, for the isolation of bacteria from marine fish.

A study on the salt dependency of the bacteria on marine fish (Simidu & Hasuo, 1968) suggested that these bacteria had different salt requirements from those of bacteria in sea water, and that basic research would be needed on the culture media for bacteria from fish. The present report is concerned with an attempt to obtain the culture medium which will give maximum viable counts on marine fish.

METHOD

Plate counts were carried out on plaice, 'Ishigarei' (*Kareius bicoloratus*) and on a flatfish, 'Hirame' (*Paralichthys olivaceus*) purchased at Funabashi Fish Market, Chiba Prefecture. In most cases, the fish were still alive at the beginning of the experiments.

A piece of skin from the mid dorsal part of the fish, 6·0 cm. in area and with or without underlying muscle was excised from the fish and homogenized with 40–100 ml. salt solution. Two different salt solutions were used for the dilutions, i.e. 0·85% sodium chloride solution and a solution containing 0·85% sodium chloride and 1·0% potassium chloride. From serial dilutions, 0·5 ml. were placed on to triplicate plates using a mechanical pipette devised by Yanagita & Maruyama (1953). Twenty ml. of agar medium which had been held at $49° \pm 0·2°$ were poured into the plate and mixed with the diluent. Up to six different media were compared in one experiment, and, in each dilution step, the time required for the whole procedure from placing

the diluent on to the first of 18 plates to the end of mixing the agar in the last plate was under 9 min. The colonies developed on the plates were counted after incubation of 3–5 days at 24–25°.

RESULTS

Concentration and composition of salts in the medium

Investigation on the salt dependency of the fish microflora (Simidu & Hasuo, 1968) showed that the highest viable counts were generally obtained at the concentration of salts corresponding to 25–75% dilution of sea water.

The fact that the ratio of potassium ion to sodium ion is about 2·7 in fish muscle (Thurston, 1958) and about 0·7 in bacteria (*E. coli*) (Guillemin & Larson, 1922) seemed to leave some doubt about the supposition that the ratio of these ions in sea water, i.e. 0·2, would be the most suitable for the growth of the bacteria which had grown or survived on marine fish.

Table 1. *Composition of salt solution used in the experiment shown in Table 2*

	Solution					
	Artificial sea-water	A	B	C	D	E
	g/1000 ml.					
NaCl	23·5	5·0	13·4	14·6	19·5	24·3
KCl	6·6	29·8	19·4	18·6	12·4	6·2
$Na_2SO_4.10H_2O$	8·9	—	9·7	—	—	—
K_2SO_4	—	5·2	—	—	—	—
$MgCl_2.6H_2O$	10·2	10·2	10·2	—	—	—
$MgSO_4.7H_2O$	—	—	—	6·2	6·2	6·2
$CaCl_2.2H_2O$	1·5	1·5	1·5	1·5	1·5	1·5
Ionic ratio of K:Na	0·2	5·0	1·0	1·0	0·5	0·2

Viable counts were compared among media which have different ratios as to the ionic concentration of sodium and potassium. The composition of the salts in these media are given in Table 1. Two different basal media were used with the salt solutions. The composition of the first one used in the experiment with flatfish was: 1·0% Polypepton (Daigo Eiyo Kagaku Co., Osaka), 0·5% Meat Extract (Kyokuto Seiyaku Kogyo Co., Tokyo) and 1·2% agar. The second one used in the experiment with plaice contained 1·0% Polypepton, 0·3% Lab-Lemco (Oxoid), 0·3% Yeast Extract (Difco) and 1·2% agar. The pH value of the media was adjusted to 7·0 in both experiments.

The results of the plate counts given in Table 2 showed that the highest counts were obtained in the media in which the ionic ratio of the sodium and potassium was 1:1.

Effect of other nutrients and pH value

The 'Meat Extract' which is widely used in Japan is composed mainly of a concentrate of a hot-water extract of fish (bonito), and is known to contain some substances inhibitory for the growth of bacteria.

The effect of Meat Extract (Kyokuto) on viable counts was compared with Lab-Lemco (Oxoid) and with Lab-Lemco + Yeast Extract (Difco). The constituents

Table 2. *Effect of salt composition of media on viable count of fish*

A. Bacterial counts from a flatfish sample

Dilution of solution (%)		Solution: Artificial sea water	A	B
		Counts × 10^{-2}/g.		
100		204	168	227
		186	138	221
		197	151	229
	Average	196	152	226
50		366	231	344
		298	243	305
		304	205	373
	Average	323	226	341

Analysis of variance

	Degrees of freedom	Sum of squares	Mean square
Dilution	1	49,928	49,928
Solution	2	28,515	14,257·5*
Interaction	2	2,317	1,158·5
Individuals	12	6,570	547·5
Total	17	87,330	—

* 14,257·5/547·5 = 26·0, significant at 1 % level.

B. Bacterial counts from a plaice sample

Dilution of solution (%)		Solution: C	D	E
		Counts × 10^{-2}/g.		
75		50	36	42
		54	51	46
		58	46	41
	Average	54	44·3	43
25		48	46	40
		53	35	39
		53	51	43
	Average	51·3	44	40·7

Analysis of variance

	Degrees of freedom	Sum of squares	Mean square
Dilution	1	14·2	14·2
Solution	2	390·1	195·1*
Interaction	2	4·8	2·4
Individuals	12	322·0	26·8
Total	17	731·1	—

* 195·1/26·8 = 7·28, significant at 1 % level.

of the media shown in Table 3 were dissolved into $\frac{1}{4}$ strength solution C (Table 1). The pH was adjusted to 7·0.

The results (Table 3) showed that the combination of 0·3% Lab-Lemco and 0·3% Yeast Extract gave the highest counts.

The growth-supporting effect of two different peptones, i.e. Trypticase (BBL) and Polypepton (Daigo) was compared at two different concentrations. The composition of the basal medium was 0·3% Lab-Lemco, 0·3% Yeast Extract and 1·2% agar.

Table 3. *Effect of extracts in media on viable counts of fish*

A. Composition of organic nutrients in media

No. of medium	Meat Extract (Kyokuto) (%)	Lab-Lemco (Oxoid) (%)	Yeast Extract (Difco) (%)	Polypepton (Daigo) (%)
1	0·5	—	—	1·0
2	—	0·5	—	1·0
3	—	0·3	0·3	1·0

B. Bacterial counts with a plaice sample

No. of medium	Counts $\times 10^{-2}$/g., average of 3 plates	Standard deviation
1	9·7*a*	2·6
2	15·0*b*	4·6
3	23·3*c*	3·3

Differences between *a* and *c*, and *b* and *c* were significant at 5% level.

Table 4. *Effect of different concentration of peptones on viable counts of fish*

Bacterial counts from a plaice sample

Concentration of peptone (%)	Peptone: Polypepton (Daigo) Counts $\times 10^{-3}$/g.	Peptone: Trypticase (BBL) Counts $\times 10^{-3}$/g.
1·0	48	56
	53	49
	53	52
	Average 51·3	52·3
0·5	41	48
	41	43
	54	42
	Average 45·3	44·3

Analysis of variance

	Degrees of freedom	Sum of squares	Mean square
Concentration	1	147·0	147·0*
Peptone	1	0	0
Interaction	1	3·0	3·0
Individuals	8	174·7	21·8
Total	11	324·7	—

* 147·0/21·8 = 6·74, significant at 5% level.

The constituents were dissolved in diluted C solution and peptones were added to the medium at concentrations of 0·5 and 1·0%. The pH value of the media was adjusted to 7·0.

The results of the plate counts are shown in Table 4. Whereas there was virtually no difference of the viable counts between two peptones, the advantage of the higher concentration was observed in both peptones.

ZoBell (1941) showed with bacteria in sea water that the highest plate counts were obtained in alkaline media and in the presence of minute amount of ferric ion.

The effect of both pH value and added ferric ion was examined with a basal medium composed of 1·0% Polypepton, 0·3% Lab-Lemco, 0·3% Yeast Extract, and 1·2% agar dissolved in a half dilution of C solution.

The results, counted after 5 days incubation at 25°, are given in Table 5. The results showed that although higher counts were obtained at higher pH ranges, the effect of pH was not as remarkable as in ZoBell's experiments with sea water. No appreciable effect was observed with the addition of ferric ion to the medium.

Table 5. *Effect of pH value and ferric ion*

Bacterial counts with a plaice sample

pH	$FeSO_4.7H_2O$ (%)	Count $\times 10^{-2}$/g. average of 3 plates	Standard deviation
6·84	—	133	5·7
7·21	—	155	3·1
7·50	—	169	26·0
7·97	—	165	13·7
7·23	0·01	163	11·9

DISCUSSION

Wood (1953), in his study on the marine environments of Eastern Australia, stated that there was no significant difference in the growth of the fish flora on freshwater and sea-water agar. Liston (1956), on the other hand, reported that higher viable counts were generally obtained from fish with sea-water medium than horse-heart infusion agar using tap water. In his experiments, however, as he noticed on the evaluation of the results on gut flora, the advantage of the higher salt content in the sea-water medium on the one hand seemed to compete with the advantage of higher nutrition in the horse heart agar on the other hand, and this made it difficult to evaluate the effect of the salts themselves.

The heterogeneity of the fish microflora growing on a salt range (Simidu & Hasuo, 1968) suggested that it would hardly be possible to obtain all the heterotrophic aerobes by using a single medium with any particular salt concentration. However, it would be not practical to use many media of different salt concentrations for the isolation and identification of fish bacteria, for it might make difficulties in the interpretation of the results.

Based on the present study, the medium (Table 6), called 'medium B' was developed for the study of the bacterial flora of marine fish. This medium can also be used for the examination of the bacteriological conditions of fish instead of for the usual plate count agar or nutrient agar with or without added 3% salt.

Table 6. *Composition of 'Medium B'*

Polypepton (Daigo)	10·0 g.	$MgSO_4.7H_2O$	3·0 g.
Lab-Lemco (Oxoid)	3·0 g.	$CaCl_2.2H_2O$	1·5 g.
Yeast Extract (Difco)	3·0 g.	Agar	12·0 g.
NaCl	8·0 g.	Tap water	1000 ml.
KCl	10·0 g.	pH 7·5	

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Microflora in the Digestive Tract of Inshore Fish in Japan

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SUMMARY

The aerobic bacterial flora of the digestive tract of jackmackerel (*Trachurus japonicus*) was found to be composed almost exclusively of species of the genus *Vibrio*. Comparison of the flora of the stomachs with that of the intestines of these fish indicated that only vibrios were found in the intestines.

INTRODUCTION

The digestive tract of marine fish carries a heavy bacterial load which plays an important role in spoilage after the death of the fish. Workers have reported quite different generic compositions of the bacterial flora of the digestive tract (Shewan, 1961). The differences could be ascribed mainly to the following three factors: (1) environmental conditions under which the fish were caught; (2) changing views of microbial classification, especially of Gram-negative rods, which are the predominant organisms in the gut flora; (3) differences in methods of isolating the bacteria. Concerning the last factor, special consideration should be given to the constitution of the media used for isolating fish bacteria; this seems particularly true for the bacteria of the digestive tract. Liston (1957) suggested that some vibrios of the digestive tract have strict nutritional requirements and hence the isolation medium should contain higher amounts of organic nutrients. A distinctively halophilic nature of the flora of the digestive tract has been found in some cases (Simidu & Hasuo, 1968*a*). The present investigation was undertaken to determine the generic composition of the aerobic bacterial flora in the digestive tract of marine fish caught in Japanese waters. An improved medium which was developed to obtain the maximal viable counts with marine fish (Simidu & Hasuo, 1968*b*) was used to isolate the bacteria.

METHODS

Jackmackerel (*Trachurus japonicus*) used in this investigation were bought at Funabashi Fish Market and at Katakai Beach, Chiba Prefecture. When bought in the market the fish had been kept in ice-water for a day after being caught. At Katakai Beach, fresh fish were obtained immediately after landing. The fish were brought to the laboratory in an icebox, about 2 hr distant from the beach. Fish were immersed in 0·5% crystal violet solution for 3 min., then washed with 98% (v/v) ethanol in water. Whole digestive tracts or separated stomachs and intestines were removed aseptically and homogenized with a diluent solution of 9 times the weight of the sample. The diluent solution used consisted of 0·8% NaCl + 1·0% KCl. Plate counts

were made on two different media (see Table 1). Colonies were counted after 5 days of incubation at 24°. Before counting, about 30 colonies were transferred from an area chosen at random on each plate of medium B to a liquid medium of the same composition (B broth). The generic composition of the isolates was determined mainly according to the scheme of Shewan, Hobbs & Hodgkiss (1960).

Table 1. *Composition of the media used*

	A (g.)	B (g.)
Polypepton (Daigo Eiyo-Kagaku)	10·0	10·0
Lab-Lemco beef extract (Oxoid)	3·0	3·0
Yeast extract (Difco)	3·0	3·0
NaCl	1·6	8·0
KCl	2·0	10·0
$MgSO_4.7H_2O$	0·6	3·0
$CaCl_2.2H_2O$	0·3	1·5
Agar	12·0	12·0
Distilled water	1000 ml.	1000 ml.

Adjusted to pH 7·5

RESULTS AND DISCUSSION

Results with whole digestive tracts of fish from the market are shown in Table 2. The average weight of the fish used for the experiment was 59 g. The present results with jackmackerel confirm previous experimental results with plaice, namely, that halophilic vibrios were predominant in the gut flora (Simidu & Hasuo, 1968*a*). Although some isolates produced gas from glucose in Barsiekow medium containing 3 % NaCl, they were classified as members of the genus *Vibrio*, based on their sensitivity to the vibriostatic compound 2,4-diamino-6,7-di-isopropylpteridine.

Table 2. *Viable counts and generic composition of aerobic bacteria from the digestive tracts of jackmackerel* (*Trachurus japonicus*)

	Sample 1	Sample 2
	Viable counts	
Medium A	$3·7 \times 10^3$	$4·0 \times 10^3$
Medium B	$1·2 \times 10^6$	$9·7 \times 10^4$
	Number of strains	
Vibrio Aerogenic	5 (5)*	4
Vibrio Non-aerogenic	15	15 (3)*
Micrococcus	0	3
Total	20	22

* Number of luminescent strains in parentheses.

The composition of the bacterial flora of the digestive tracts differed clearly from that of the sea water of the coastal area (Simidu & Aiso, 1962) and that of the surface slime of the fish (T. Haraguchi,, personal communication). Both in sea water and

in fish slime, large numbers of pseudomonad and achromobacters were found in addition to vibrios.

In the next experiment with fresh jackmackerel obtained at Katakai Beach, the flora of stomachs was compared with that of intestines. The average weight of the fish was 43 g. The digestive tracts of the fish were removed aseptically, and the stomachs and intestines separated. Materials from six fish whose stomachs did not contain food were mixed and homogenized with the diluent solution. The results of the plate counts given in Table 3 show that a peculiar flora which was different from that of the stomachs was found in the intestines.

Table 3. *Viable counts and generic composition of the aerobic bacteria in the stomachs and intestines of jackmackerel*

	Stomachs	Intestines	Intestines*
	Viable counts (no./g. sample)		
Medium A	$2{\cdot}4 \times 10^5$	$2{\cdot}9 \times 10^5$	$1{\cdot}2 \times 10^4$
Medium B	$2{\cdot}6 \times 10^5$	$4{\cdot}6 \times 10^6$	$1{\cdot}2 \times 10^6$
	Number of strains		
Vibrio Aerogenic	2 (2)†	9 (2)†	13 (1)†
Vibrio Non-aerogenic	6 (0)†	17 (6)†	16 (13)†
Pseudomonas	2	1	0
Achromobacter	7	0	0
Flavobacterium	2	0	0
Sarcina	7	0	0
Corynebacterium	3	0	0
Bacillus	1	0	0
Total	30	27	29

* Result with the fish stored at 2° for 21 hr.
† Number of luminescent strains in parentheses.

Other than anaerobic conditions, several factors which favour the selective growth of vibrios in the intestines might be considered. One of these factors is the resistance of vibrios to the effect of bile salts. Table 4 shows the results of an experiment on the effect of bile on the growth of isolates from stomachs and intestines. Test organisms were grown in B broth for 24 hr at 25°, and then streaked on plates of B agar to which different amounts of Oxgall (Difco) had been added. The growth of the organisms was examined after 5 days of incubation at 25°.

Table 4. *Sensitivity of isolates to bile*

Concentration of Oxgall (Difco) in the medium (%)	Isolates from: Stomachs	Isolates from: Intestines
	No. of strains grown at the given concentration of bile	
0·0	27	23
0·1	26	22
0·3	18	22
1·0	17	22
2·5	15	22
10·0	15	22

Thjøtta & Sømme (1943), in their survey of bacteria of marine and freshwater fish, observed that while vibrios predominated in the flora of both surface and gut of fish from the west coast of Norway, other genera such as *Bacterium*, *Achromobacter* and *Escherichia* were the main constituents of the gut flora of fish from Oslofjord. Most of the strains of the dominant genus, *Bacterium*, were described as having polar flagella and producing acid from glucose, which suggests that some of them may have been vibrios according to the present classification.

Liston (1957) showed that the digestive tract of lemon sole and skate from the North Sea contained large numbers of vibrios, which constituted about 35–74% of the total aerobic flora throughout his continuous 27-month period of observation. In our experiments, the gut floras of plaice and jackmackerel consisted almost exclusively of vibrios. The differences in composition of the media used for isolation as well as regional difference may have been responsible for the quantitative differences.

The present results strongly suggest that the indigenous bacterial flora of the intestine of marine fish consists only of vibrios. This fact has aetiological significance for fish diseases in fish-farming ponds, as well as in food poisoning caused by *Vibrio parahaemolyticus*, which account for about half the total number of food poisoning in Japan.

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Cellular Lipid and the Antistaphylococcal Activity of Phenols

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SUMMARY

Staphylococcus aureus (OXFORD) when grown in the presence of glycerol increased its cellular lipid content to the extent of 18% calculated on a cell dry-weight basis. When challenged by a homologous series of 4-*n*-alkyl phenols (H- to C_6H_{13}-) an increase in resistance was noted with the glycerol grown (fattened) cocci after the 4-*n*-butyl (C_4H_9-) phenol. This phenomenon was investigated by means of electrophoresis of single cocci, the measurement of drug uptake, and the effect on leakage of cytoplasmic constituents as well as by the assessment of minimum inhibitory concentrations. Electron micrographs of thin sections of normal and fattened cocci were also examined.

INTRODUCTION

Hugo & Stretton (1966*a*, *b*) showed that when the lipid content of certain Gram-positive organisms was increased by growth in the presence of glycerol, these fattened organisms became more resistant to the penicillins, quinacillin, benzylpenicillin, methicillin and cloxacillin; in some cases, when normal lipid content of the organisms was decreased by treatment with lipase, the resistance decreased. It was decided to investigate this phenomenon further but to challenge both fattened and normal organisms with a homologous series of compounds, the 4-alkyl phenols, because the physical properties of this series might be expected to vary in a regular and determinable or determined pattern. Previous findings had shown that the lipid content of the Oxford staphylococcus could be increased threefold on growth in the presence of glycerol; accordingly, this organism was used. Some of the results reported here were briefly reported elsewhere (Hugo & Franklin, 1966).

METHODS

Organism. The organism used throughout this work was *Staphylococcus aureus*, strain OXFORD.

Materials. 4-Methylphenol (*p*-cresol) and oleyl alcohol were of laboratory reagent grade (British Drug Houses Ltd.); *p-n*-butylphenol and *p-n*-pentyl (amyl) phenol were gifts from Boots Pure Drug Co. Ltd., Nottingham; the remaining phenols were from Kodak Ltd., Kirby, Liverpool. All other chemicals were of analytical reagent quality.

The phenols were purified by distillation, yielding white crystalline products and were stored in sealed amber glass ampoules, from which samples were removed as required. Melting points (uncorrected) of the products so purified were: phenol 43°, 4-methylphenol (*p*-cresol) 34°, 4-ethylphenol 47–48°, 4-*n*-propylphenol 21–22°, 4-*n*-butylphenol 22°, 4-*sec.*-butylphenol 59°, 4-*n*-pentylphenol (amylphenol) 87–89°, 4-*tert.*-amylphenol 92–93°, 4-*n*-hexylphenol 23–24°.

Media and conditions of growth. Culture media were prepared from Oxoid material and contained (g./l.): peptone (Oxoid no. 1) 10, meat extract (Lemco, Oxoid) 5, sodium chloride 5, and, where appropriate, glycerol 30. Sterilization was done at 115° for 30 min.; final pH 7·4.

Buffer solutions. Phosphate buffer was prepared by mixing appropriate quantities of $Na_2HPO_4 12H_2O$ and $KH_2PO_4 2H_2O$, giving a range from pH 5·4 to 8·0. Tris buffer was used when cellular inorganic phosphate was being investigated. Carbonate + bicarbonate buffer was used in one experiment for the range pH 8–10·6.

Determination of minimum inhibitory concentrations. A tube dilution method was used with inoculum to give finally 10^6 cocci/ml. Results were read after 3 days.

Total lipid and electrophoretic mobilities of cocci. These were determined as previously described (Hugo & Stretton, 1966*a*).

Analytical determination of phenols. This was by the colorimetric method of Lykken, Treseder & Zahn (1946), which involves the conversion of the phenols to nitrosophenols and the determination of the coloured nitrosophenol spectroscopically with a Unicam SP 600 (Cambridge Instruments Ltd.). For each phenol, a calibration curve was constructed from which the phenol content of unknown solutions was calculated. When phenols were to be determined in aqueous solution uncontaminated by cellular constituents the appropriate D value ($\ln I_0 I$) was determined in the ultraviolet region at the previously determined λ_{max} using the SP 500 spectrophotometer (Cambridge Instruments Ltd.). The λ_{max} values lay between 270 and 280 mμ.

Water solubility of phenols. Water was saturated with the appropriate phenol which was determined spectroscopically. From the optical density of the saturated solution and a solution of known concentration at the appropriate λ_{max} the water solubilities were calculated. Values obtained (g./100 ml.) were: phenol, 9·37; 4-methylphenol, 2·54; 4-ethylphenol, 0·72; 4-propylphenol 0·36; 4-*n*-butylphenol, 0·10; 4-*n*-pentylphenol, 0·044; 4-*n*-hexylphenol. 0·045; 4-*sec.*-butylphenol, 0·12, 4-*tert.*-amylphenol, 0·033.

Distribution coefficients. These were obtained as between oleyl alcohol and 0·067 M-phosphate buffer by adding known volumes of the alcohol and the phenol in the buffer and rotating in a stoppered tube at room temperature until equilibrium was established. A sample of the aqueous layer was assayed for its phenol content spectrophotometrically and the partition coefficient calculated from the following expression:

$$\text{distribution coefficient} = \frac{\text{Volume of aqueous phase} \times \text{final concentration in oily phase}}{\text{Volume of oily phase} \times \text{final concentration in the aqueous phase}}$$

The phosphate buffer held the pH value at 7·4 but was of a sufficiently high dilution to ensure that the ions did not contribute significantly to the distribution. Phenol concentrations in the initial aqueous layer were of the same order as encountered in the antibacterial studies. Because of the high solubility of the higher members in the oily phase, their concentration in the aqueous phase was too low for reproducible results to be obtained.

Determination of uptake of phenols by Staphylococcus aureus. To 18 ml. of the phenol solution was added 2 ml. of the bacterial suspension (equiv. 10 mg. dry wt/ml.) in 0·067 M-tris buffer (pH 7·4). The mixtures were maintained in a water bath at 37° and at appropriate times the bacteria were removed by centrifugation twice (15 min. each) at 9000*g* and the residual phenol determined by the colorimetric method.

Determination of phenol-induced leakage of cellular constituents. Determination of leakage of cellular material absorbing at 260 mμ is complicated by the 270–280 mμ absorbing peak of the phenols. However, by extraction of the supernatant liquid remaining after centrifugation of phenol-treated cocci with three 25 ml. amounts of chloroform, the phenols (except phenol itself) were removed and the aqueous layer after warming to remove dissolved chloroform was examined for material absorbing at 260 mμ. The adequacy of this procedure was tested by mixing the supernatant fluid from boiled cocci, of which the 260 mμ absorbing material had been determined, with a solution of each of the phenols in turn, subjecting this mixture to the extraction procedure and redetermining the 260 mμ absorbing material; almost quantitative recoveries were obtained. Leakage of cytoplasmic constituents was also investigated by determining inorganic phosphate in the cell-free supernatant fluid by the method of Fiske & SubbaRow (1925). Phenols, at the concentrations used, did not interfere in this determination.

Preparation of thin sections of cocci and electron microscopy. This was done according to the method described by Hamilton & Stubbs (1967), with the glutaraldehyde + OsO_4 fixative.

RESULTS

Growth of cocci in glycerol and their lipid content

Ten serial subcultures of the Oxford staphylococcus in the culture medium containing 3% glycerol resulted in a total lipid content of 18·8 ± 0·8; the lipid content of cocci grown under similar circumstances but without glycerol was 6·5% ± 0·4, in good agreement with the findings of Hugo & Stretton (1966*a*, *b*). Growth curves showed that the presence of glycerol had no significant effect on the log phase or the mean generation time of this organism but the final yield of organisms was higher with glycerol. Because fattened cocci rapidly revert to their normal lipid content after one subculture in the absence of glycerol, all experiments with fattened cocci involving growth were done in the presence of glycerol, 3% (Fig. 1).

Table 1. *Minimum inhibitory concentrations for control and fattened cocci of Staphylococcus aureus* (*OXFORD*)

	Control cocci	Fattened cocci
	m.i.c. (μg./ml.)	
Phenol	2200	2200
Cresol	1300	1300
Ethylphenol	640	640
Propylphenol	390	390
n-Butylphenol	85	90
n-Amylphenol	38	44
n-Hexylphenol	42	52
sec.-Butylphenol	140	150
tert.-Amylphenol	55	110

Minimum inhibitory concentrations of phenols

These are presented in Table 1. The final inoculum was 10^6 cocci/ml. As the tubes inoculated with fattened cocci must contain 3% glycerol and in view of the findings of Cooper (1948) that glycerol in concentrations of 30–40% depresses the antibacterial

effects of phenols to the extent of 20–40%, experiments were made on the effect of 3% glycerol on the minimum inhibitory concentration (m.i.c.) values of the phenols used. At this concentration, glycerol had no detectable effect.

Uptake of phenols by normal and fattened cocci

Preliminary experiments showed that equilibrium between cocci and the phenol was rapidly attained and was virtually complete in 5 min. The data obtained is shown in Table 2, where it can be seen that the fattened cocci took up slightly less of the phenols tested than did the controls.

Table 2. *The uptake of phenols by fattened and unfattened (normal) cocci of Staphylococcus aureus (OXFORD)*

Phenol	Uptake of phenol, μg/mg dry wt. cells	
	Fattened cells	Unfattened cells
Propyl	42·9	46·4
n-Butyl	22·2	27·3
n-Amyl	34·8	49·9
sec.-Butyl	84·8	96·3
tert.-Amyl	41·8	50·8

Table 3. *Staphylococcus aureus (OXFORD). Increase in electrophoretic mobilities as compared with mobilities in buffer of control cocci (a) and fattened cocci (b) in the presence of various concentrations of nine p-alkyl phenols in phosphate buffer (pH 7·4, I = 0·01)*

		Phenol			4-Methylphenol			4-Ethylphenol		
Concentration (μg./ml.)		1000	2000	3000	1000	1500	2000	400	600	800
Increase in mobilities (μ/sec./V./cm.)	(a)	0·07	0·29	0·33	0·52	0·55	0·60	0·55	0·70	0·82
	(b)	0·51	0·64	0·69	0·76	0·77	0·84	0·80	0·92	1·04
		4-Propylphenol			4-Butylphenol			4-Pentylphenol		
Concentration (μg./ml.)		200	400	500	50	100	150	20	50	80
Increase in mobility (μ/sec./V./cm.)	(a)	0·56	0·67	0·94	0·79	1·04	1·48	0·52	0·72	1·17
	(b)	0·80	0·90	1·16	1·02	1·46	1·82	0·76	1·13	1·43
		4-Hexylphenol			4-*sec.*-Butylphenol			4-*tert.*-Pentylphenol		
Concentration (μg./ml.)		30	60	75	100	150	200	50	100	125
Increase in mobility (μ/sec./V./cm.)	(a)	1·15	1·43	1·53	0·78	0·90	1·07	0·56	0·75	0·96
	(b)	1·46	1·66	1·86	1·05	1·17	1·31	0·79	0·99	1·28

Electrophoretic mobilities

Mobilities in the presence of sodium dodecylsulphate (SDS). Data obtained in the presence and absence of SDS (10^{-6}, 10^{-5}, 10^{-4} M) gave figures of a very similar order to that obtained by Hugo & Stretton (1966*a*) for the Oxford staphylococcus and confirmed the build-up of surface lipid in the presence of glycerol and its rapid depletion after one subculture in its absence (Fig. 1).

Mobilities of fattened and unfattened cocci in the absence of added agent. The

absolute mobility, μ/sec./V./cm. measured on the same day of fattened cocci was 2·002 and of control cells, 2·239, a difference of 10·2%.

Mobilities of fattened and unfattened cells in the presence of phenols. Mobilities were measured at three concentrations of each phenol. The data are presented in Table 3, where it can be seen that whereas the phenols in general increase the negative mobility of the cocci the increase was greater with fattened cocci. The effect of increasing chain length on the mobility differences is shown in Fig. 2. Similar figures were obtained at different initial concentrations of the phenol.

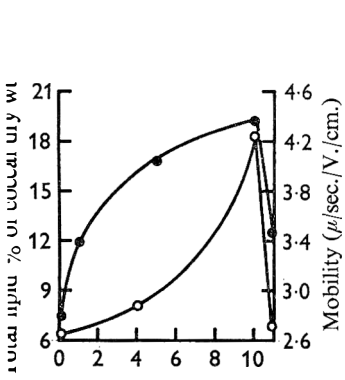

Fig. 1

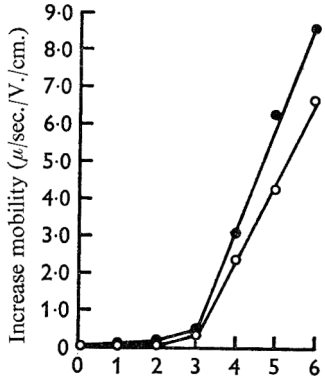

Fig. 2

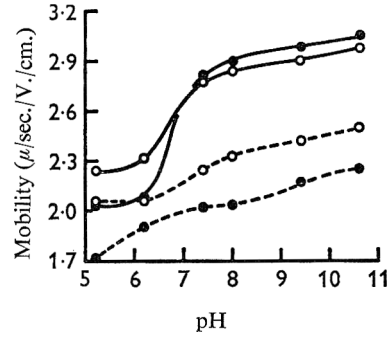

Fig. 3

Fig. 1. The build-up of cellular lipid in *Staphylococcus aureus* (OXFORD) on 10 successive subcultures in nutrient broth containing 3% glycerol and its depletion after one subculture in the absence of glycerol. Total lipid obtained by extraction, —○——○—; electrophoretic mobility in the presence of 10^{-4} M-SDS, ●—●.

Fig. 2. Effect of chain length (number of carbon atoms in the 4-alkyl phenol substituent) on the electrophoretic mobility of *Staphylococcus aureus* (OXFORD) cocci. Fattened cocci, ●—●; control cocci, ○—○.

Fig. 3. The effect of pH value on the electrophoretic mobilities of *Staphylococcus aureus* (OXFORD) cocci in the presence and absence of 4-ethylphenol (400 μg./ml.). Control cocci in buffer, ○ - - - - ○; fattened cocci in buffer, ● - - - ●; control cocci in ethylphenol solution, ○——○; fattened cocci in ethylphenol solution, ●——●.

Effect of pH on the mobilities of cocci in the presence of phenols. For the range pH 5–8 phosphate buffer was used and for pH 8–10·6 carbonate+bicarbonate buffer. Phenol solution, buffer solution and coccal suspensions were mixed so that the final ionic strength of the system was 0·01, and the coccal density equiv. 0·1 mg. dry wt/ml. Similar patterns were found for each of the phenols. The data for *p*-ethylphenol are shown in Fig. 3. This experiment indicated the necessity for controlling the pH value of the system during electrophoretic studies, for small changes in pH value over the range pH 6–8 result in marked changes in mobilities.

Phenol-induced leakage of cellular constituents from fattened and unfattened cocci

When the effect of time on leakage of 260 mμ absorbing material and inorganic phosphate was examined, it was found that a rapid initial leakage of both constituents occurred within 2·5 min. In the case of the lower members of the phenol series (up to the propyl compound) it was complete in 30 min.; in the remainder it slowly increased

up to 180 min. When the data for fattened and unfattened cocci are compared it can be seen that there was a rather greater release of these materials from the unfattened cocci. This is illustrated in Table 4, where the leakage of the two cytoplasmic constituents after 15 min. contact with the phenols is presented.

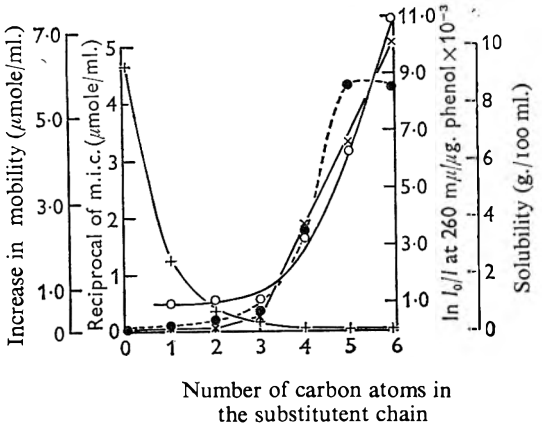

Fig. 4

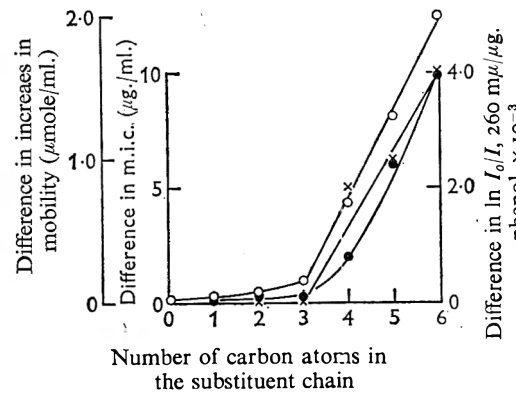

Fig. 5

Fig. 4. *Staphylococcus aureus* (OXFORD). Effect of chain length (number of carbon atoms) in 4-alkyl substituent on: electrophoretic mobility, ×——×; m.i.c. plotted reciprocally, ●- - - -●; leakage of 260 mμ absorbing material, ○——○; water solubility, + – – – +.

Fig. 5. *Staphylococcus aureus* (OXFORD). Effect of chain length on the difference in observed effect as between control and fattened cocci as measured by: m.i.c. plotted reciprocally, ×——×; electrophoretic mobility, ○——○; leakage of 260 mμ absorbing material, ●——●.

Table 4. *Staphylococcus aureus OXFORD. The release of inorganic phosphate (Pi) and 260 mμ absorbing material from normal and fattened cocci after treatment for 15 min. with phenols at the concentrations stated*

	Concn. phenol (μg./ml.)	μg Pi/mg. dry wt. cocci		ln I^0/I (260 mμ)/mg. dry wt. cocci	
		Control	Fattened	Control	Fattened
Phenol	500	0·44	0·41	—	—
4-Methylphenol	250	0·32	0·18	0·24	0·13
4-Ethylphenol	120	0·33	0·28	0·13	0·11
4-Propylphenol	200	0·28	0·22	0·21	0·20
4-Butylphenol	100	0·53	0·36	0·33	0·25
4-Pentylphenol	80	2·78	2·07	0·50	0·30
4-Hexylphenol	60	3·33	3·07	0·66	0·43
4-*sec.*-Butylphenol	200	0·63	0·60	0·45	0·41
4-*tert.*-Pentylphenol	100	0·80	0·60	0·40	0·27

Electron micrographs

Thin sections of the Oxford staphylococcus grown for 10 generations in the presence of glycerol showed a distinct thickening of the extra-cytoplasmic layers (Plate 1). The thickening appeared to be confined to the walls and was especially pronounced in the septum separating the dividing cocci where presumably active cell-wall synthesis was occurring.

General

The inter-relationship of the several criteria and properties used in this work to evaluate coccal interaction are convincingly seen when the results are plotted on one graph as a function of the number of carbon atoms in the 4-primary alkyl substituent. In the first such plot (Fig. 4) the general effect of increasing the number of carbon atoms on the antibacterial and auxiliary activities and properties of phenols upon normal cocci is shown. It is clear that the side chain effect makes itself felt after the C_3 (propyl) phenol.

The influence of cellular lipid on the m.i.c., mobility and cytoplasmic integrity as it changes with chain length of the phenols was shown by plotting differences in response at each experimental point as between normal and fattened cocci against alkyl chain length (Fig. 5). Once again the differences become marked after the $-C_3$ compound, suggesting that lipid begins to exert its protective effect when the phenol tends to become more lipid soluble.

DISCUSSION

It is apparent from evidence from various sources that variation in the level of cellular lipid may be one of the factors involved in bacterial resistance (Chaplin, 1952; Church, Halvorson & Ramsey, 1956; Lowick & James, 1957; Vaczi & Farkas, 1961; Ivanov, Markov, Golowinsky & Charisanova, 1964; Truby & Bennett, 1961).

The use of a homologous series of phenols, such as the 4-normal alkyl phenols, a family of compounds whose properties change in a fairly regular way, with regard to water solubility and distribution coefficient between an aqueous and oily phase, might prove to be an ideal tool to test systematically the effect, if any, of cellular lipid upon resistance.

In considering the data presented in this paper it is reasonable to suppose that the phenols will distribute themselves between the lipid and aqueous components in the cell, in accordance with the normal partition laws, and in the case of fattened cells presumably these phenols will be partially distributed in the deposited lipid.

As the length of the normal alkyl side chain at position 4 increases, not only does the solubility in water decrease and the solubility in lipid increase, but the molecule tends to become polar and consequently surface active. This in turn means that it tends to orientate at an oil/water interface if such an interface is available. In fact changes in response to the phenols as between fattened and normal cells first show themselves distinctly with *n*-butylphenol. In the case of the fattened cell it is likely that, as lipid solubility increases, phenol is immobilized with the alkyl side chain immersed in the cell lipid and the phenolic hydroxy group projecting into the aqueous environment and it could well be that many drug molecules are thus trapped and sensitive areas in the cytoplasmic membrane protected.

This hypothesis receives support from the experimental data obtained. Thus, the smaller amount of material which leaks from fattened as against unfattened cells, which begins to be significant after the butyl compound, is compatible with the hypothesis that the membrane or sensitive areas in it are in some way protected. This, in turn, would explain why, when ability to reproduce (the m.i.c. value) is being measured, the fattened cells are less susceptible to the same dose of drug (above the C_4 compound) than are the control cells, for several groups of workers have equated

one toxic manifestation of non-specific antibacterial drugs with their ability to impair the integrity of the cytoplasmic membrane (Gale & Taylor, 1947; Salton, 1951; Beckett, Patki & Robinson, 1959; Hugo & Longworth, 1964).

The phenols at equivalent concentration cause an apparent increase in the mobility of the fattened cells as compared with the control cells which is here first noticeable with the C2 compound but again shows a marked difference after the C4. With the C4 compound and thereafter, more molecules are held at the cell surface with the phenolic group projecting into the aqueous fluid in which the cells are suspended. There will be a tendency for these to have a slightly greater negative charge at the surface hence the increased velocity towards the positive electrode for the same applied potential; indeed the phenols here are playing a similar role to that of SDS used as a detector of surface lipid, although of course their degree of ionization is much less.

It can be seen from Fig. 2 that differences between mobilities for fattened and control cells begin to show themselves at the C2 compound; electrophoresis is probably the most sensitive of the methods used in this work to detect drug/cell interaction. It is possible that small differences in response to all the phenols occur, but are only really apparent when they assume an order of magnitude detectable by the experimental method being used. It could well be that m.i.c. measurements, measurements of leakage of metabolites and drug uptake determinations are not of sufficient sensitivity to demonstrate any differences with lower alkyl phenols.

Finally the hypothesis of drug trapping will explain why fattened cells appear to take up less drug than do control cells, a finding which may appear at first sight to contradict the other data obtained. For the experiments used to determine drug uptake cannot distinguish between surface adsorption and general absorption; with the lipid present in the cell, fewer drug molecules penetrate into the cell and the surface becomes saturated, whereas with a normal cell many of the drug molecules taken up will pass into the interior of the cell as well as being held near the surface.

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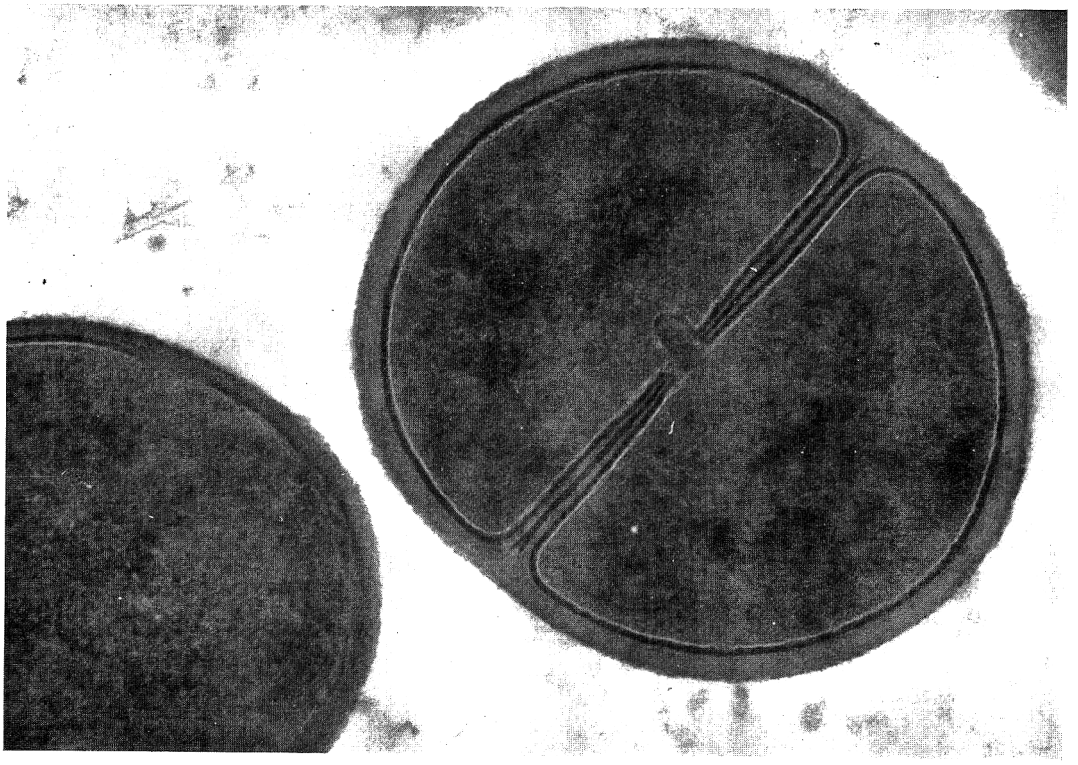

Fig. 1

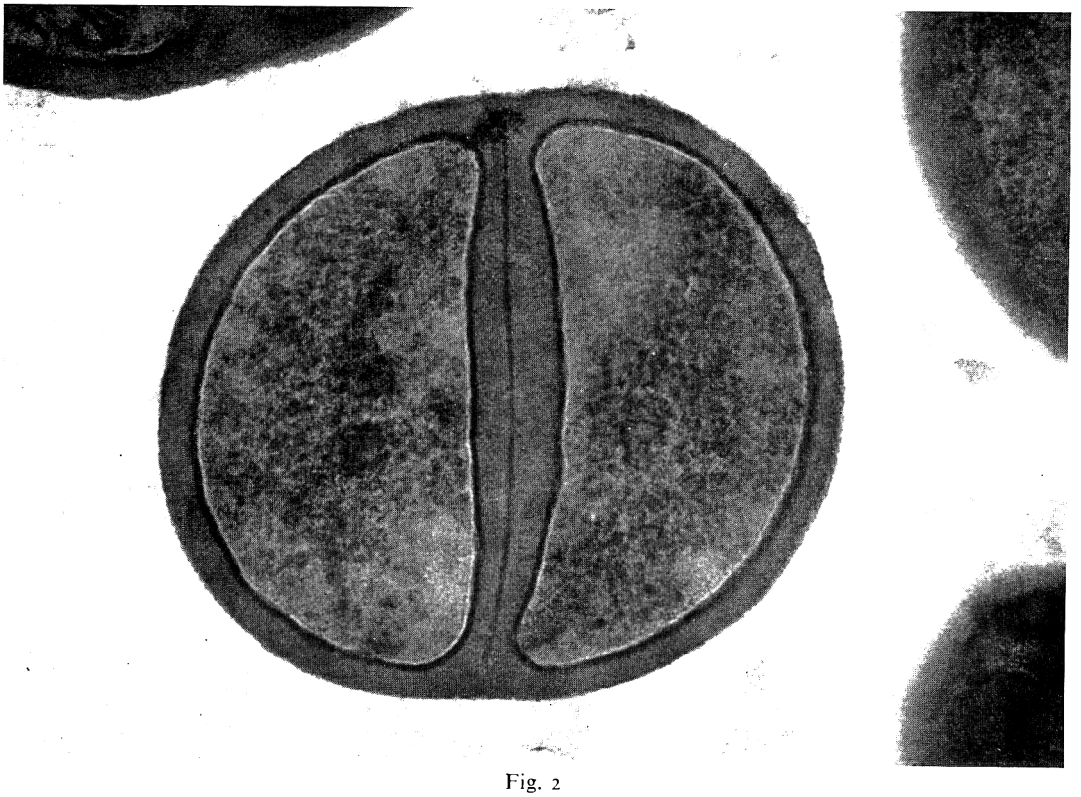

Fig. 2

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EXPLANATION OF PLATE

Fig. 1. Thin section of *Staphylococcus aureus* (OXFORD) grown in nutrient broth. × 30,000.

Fig. 2. Thin section of *Staphylococcus aureus* (OXFORD) after growth for ten generations in nutrient broth containing 3% glycerol. × 30,000.

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Toxicity of Hyperbaric Oxygen to Bacteria in Relation to the Cell Cycle and Catalase Synthesis

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(*Accepted for publication* 26 *January* 1968)

SUMMARY

Growth of *Bacillus subtilis* and of *Escherichia coli* following treatment with oxygen at 10 atmospheres for 18 hr was synchronous. Treatment at lower pressures resulted in a much lower degree of synchrony. After exposure to high-pressure oxygen to induce synchronous growth of *B. subtilis* the resistance of various stages of the cell cycle to further treatment with oxygen at 10 atm. was studied. Maximum resistance occurred immediately following division of the bacilli and then fell to a low value. The region of maximum resistance in *B. subtilis* compared fairly closely with the time of maximum catalase activity.

INTRODUCTION

Pritchard & Hudson (1967) showed that induction of catalase occurred in cultures of *Mucor racemosus* and *Candida utilis* when exposed to high-pressure oxygen (HPO) for periods of up to 20 days. It was suggested that this might provide a mechanism for preventing peroxide accumulation, thus inducing resistance to high pressure oxygen toxicity. The results presented in the present paper describe the changes in catalase activity of *Bacillus subtilis* when exposed to 10 atm. oxygen and the relation between resistance and physiological state at the time of pressure increase.

METHODS

Bacterial strains. Bacillus subtilis NCIB 3610 and *Escherichia coli* B 13 were obtained from the culture collection of the Department.

Media and cultivation. Bacteria were maintained on malt agar at 25°. Cultures for HPO treatment were grown in 25 ml. liquid glucose glutamate medium (Pritchard, 1965) in 100 ml. Erlenmeyer flasks at 37° on a rotary shaker. For *Escherichia coli* the medium was supplemented with 0·5 % (w/v) casein hydrolysate.

Catalase determination. Bacteria for catalase determination were sedimented by centrifugation, resuspended in 4 ml. cold 0·05 M-phosphate buffer (pH 6·8) and disrupted by ultrasonic treatment. The material was centrifuged for 10 min. at 30,000 *g* and the catalase activity of the supernatant fluid determined by the method of Herbert (1954). Enzyme activity is expressed per g. extracted protein.

Protein determination. Soluble protein was determined by the Folin phenol method of Lowry, Rosebrough, Farr & Randall (1951).

Bacterial counts. Total counts were made by using a bacterial counting chamber, 0·02 mm. deep, with Thoma ruling.

Viable counts. Survival following HPO treatment was determined by dispensing in

triplicate, 0·2 ml. samples of serially diluted culture on to malt agar in Petri dishes. Colonies were counted after 24 hr. incubation at 37°.

Pressure vessels. Cultures were subjected to pressure in steel pressure vessels similar to those described by Caldwell (1956).

RESULTS

Survival and catalase activity of Bacillus subtilis cultures subjected to HPO

Cultures (6 hr) were subjected to 10 atm. HPO and samples assayed for catalase activity after 24 and 48 hr exposure. There was a decrease in catalase activity after 24 hr, though by 48 hr the value had risen again. In a second experiment catalase activity was determined after 24, 72 and 144 hr exposures. There was again a decrease

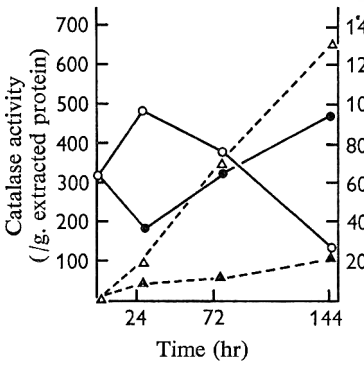


Fig. 1

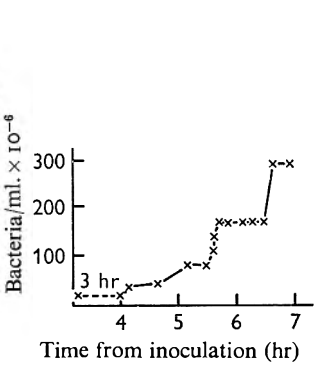


Fig. 2

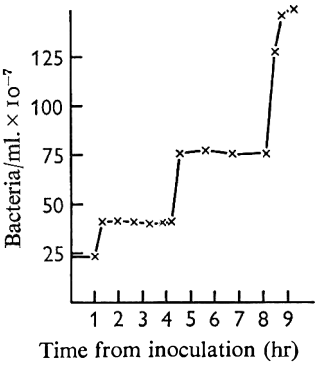


Fig. 3

Fig. 1. Effect of treatment with 10 atm. pure oxygen on catalase activity and growth of *Bacillus subtilis*. ○—○, Catalase activity in air; ●—●, catalase activity under oxygen; △ - - △, growth in air; ▲ - - ▲, growth under oxygen.

Fig. 2. Growth of *Bacillus subtilis* in air, following 18 hr exposure to oxygen at 10 atm.

Fig. 3. Growth of *Escherichia coli* in air, following 18 hr exposure to oxygen at 10 atm.

in activity in the first 24 hr, followed by a continued increase (Fig. 1). Bacterial numbers, as determined by viable counts, showed a steady increase under HPO and indicated that after 48 hr the catalase content per organism was considerably higher than that of the control organisms grown in air. In a similar experiment with an 18 hr culture almost complete lysis occurred after 24 hr, thus preventing the determination of cellular catalase activity. The same result was obtained when the experiment was repeated.

Synchrony of growth following HPO treatment

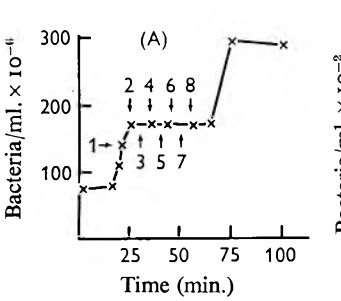
Cultures (6 hr) of *Bacillus subtilis* were subjected to oxygen at 2·5, 5·0, 7·5 and 10 atm. for 18 hr and their growth upon subsequent inoculation into 300 ml. of aerated medium at 37° followed by direct counts. Following treatment with oxygen at 10 atm., but not at the lower pressures, growth was synchronous (Fig. 2); this was confirmed in other experiments at 10 atm. It was noted that some individuals elongated but did not divide. Growth upon inoculation into fresh medium was not immediate but began after a lag period of about 3 hr. After exposure to oxygen at pressures lower than 10 atm. growth was not markedly synchronous although at

7·5 atm. the growth curve did exhibit some indication of synchrony. The lag period was shorter at the lower pressures being about 1·5 hr at 5 atm. and 7·5 atm. and virtually absent after treatment at 2·5 atm.

Treatment at 10 atm. was also done with a 24 hr culture of *Escherichia coli*. Growth upon return to air was again synchronous, apparently without a lag period (Fig. 3).

Resistance of various stages of the cell cycle to HPO

Samples were taken at 5 min. intervals during one generation of an oxygen-induced synchronous culture and placed under oxygen at 10 atm. In order to expose the bacteria as quickly as possible to the effects of HPO, 5 ml. samples were placed into 100 ml. Erlenmeyer flasks and the pressure raised in 10–15 sec. After 18 hr the pressure was released and survival determined by plate counts. All experiments showed that



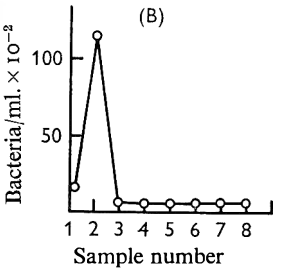


Fig. 4

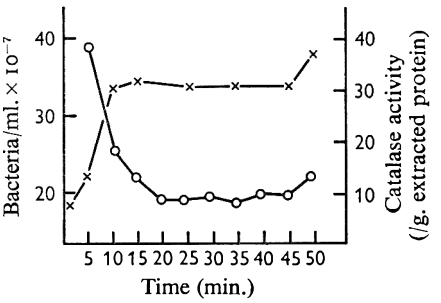


Fig. 5

Fig. 4. Survival of *Bacillus subtilis* exposed to HPO at different stages of the cell cycle. (A) Initial population showing when samples were taken for treatment with oxygen at 10 atm. for 18 hr. (B) Survival (by plate counts) following treatment.

Fig. 5. Catalase activity in relation to the cell cycle. ○—○, Catalase activity. ×—×, Bacterial numbers.

maximum survival was restricted to the early part of the cell cycle immediately following division (Fig. 4), though there was a discrepancy between the maximum number of organisms surviving the HPO treatment (*c.* 10^4/ml.) and the number submitted to the treatment (*c.* 10^8/ml.). This shows that the period, within one generation, over which bacteria are able to survive a rapid increase in oxygen pressure is so short that even in the synchronized population a minority of the bacteria were in the resistant state.

Catalase synthesis in Bacillus subtilis in relation to the cell cycle

An oxygen-induced synchronous culture of *Bacillus subtilis* was used to determine the time of catalase synthesis under atmospheric oxygen conditions; 25 ml. samples were withdrawn and chilled to stop metabolism. Because of the slow rate of cooling used for the early samples the cessation of metabolism of these samples was not immediate. Thus, by the time of ultrasonic treatment to extract the catalase, the bacteria would have advanced further in the cell cycle than was indicated by the sampling time. Thus the high value for catalase in sample 1 (Fig. 5) was probably present in the bacteria just after division rather than during division as the growth curve indicated. In the last four samples a much more rapid rate of cooling was used;

here the catalase value just began to increase by the start of the second division. Thus the synthesis of catalase occurred early in the cell cycle near the time of maximum resistance to HPO treatment.

DISCUSSION

The increased value for catalase per bacterium after 48 hr exposure to high-pressure oxygen (HPO) confirmed previous findings (Pritchard & Hudson, 1967); however, the decrease after 24 hr was unexpected. Furthermore, the lysis of bacteria in the 18 hr cultures subjected to 10 atm. seemed at variance with earlier experiments (Caldwell, 1965). A partial explanation of these results might be that the bacteria were resistant to the toxic effects of HPO treatment only when in a certain physiological state. Thus in the 18 hr culture in which growth, as indicated by turbidity, was well into the stationary phase, the majority of the bacteria would have been physiologically old. In an actively growing culture only a proportion of the bacteria would be in a resistant state; those not in this state would be killed and there would be an initial decrease in numbers. If the resistant physiological state represents a sufficiently small part of the cell cycle then the growth of an oxygen-treated culture should, on return to air, show some degree of synchrony. Experiments in which growing cultures of *Bacillus subtilis* and *Escherichia coli* were treated with oxygen at 10 atm. showed this to be the case. The fairly close correspondence between the time of maximum resistance and the time of maximum catalase activity, in *B. subtilis*, supports the hypothesis that catalase forms part of the mechanism of resistance.

Since the mechanism of resistance to HPO is suggested to be enzymic in nature it seems probable that the variation in physiological state is a result of the periodicity of enzyme synthesis. It has been demonstrated that the basal enzyme synthesis of some bacteria is ordered and related to gene position (Masters & Pardee, 1965), though enzymes of *Bacillus subtilis* and *Escherichia coli* may be induced at any time during the cell cycle (Kuempel, Masters & Pardee, 1965; Masters & Donachie, 1966; Masters, Kuempel & Pardee, 1964). Therefore survival of bacteria may be dependent on the active synthesis or presence of a high level of protective enzymes at the time of pressure increase, death of non-resistant bacteria resulting from an irreversible lesion induced more rapidly than, or preventing, the synthesis of protective enzymes.

Further experiments are being conducted to define more closely the role of catalase and investigate the effect of HPO on organisms showing true periodicity of enzyme synthesis.

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An Ecological Survey of Hydrocarbon-oxidizing Micro-organisms

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SUMMARY

An ecological survey of the microflora of an upland moorland soil and the underlying shale was made over a 2-year period. Samples were taken at different depths of about 5, 20 and 40 cm. in the soil, and from the underlying shale band in a cave system nearby, and the average total counts of bacteria and fungi were 35×10^6, 3×10^6 and 1×10^6 per g. dry wt respectively. Hydrocarbon-oxidizing organisms occurred in all the samples. The numbers and activity of these organisms were determined in a variety of ways and finally expressed as a function of the total population of each sample. A significantly higher proportion of the population of the 20 cm. soil sample was able to utilize hydrocarbons as sole energy and carbon source, than of any of the other samples. When hydrocarbons were added there was a stimulation in respiration of all samples. Maximum stimulation of respiration occurred in the 20 cm. soil sample. The E_h of the 20 cm. soil sample was lower than in any other sample, it also contained a higher level of lipid and hydrocarbons. It is suggested that the higher level of hydrocarbon oxidation in the 20 cm. soil sample is due to the accumulation of hydrocarbons with a resulting adaptation of the microbial populations. More organisms were able to oxidize the longer chain *n*-aliphatic hydrocarbons than the short chain *n*-aliphatics, aromatic and alicyclic hydrocarbons. A list of the genera, including fungi, responsible for the oxidation of each hydrocarbon is given: the fungi play an important role in the hydrocarbon-oxidizing activity of each sample.

INTRODUCTION

The oxidation of hydrocarbons by micro-organisms has been well documented (Johnson, 1964; Van der Linden & Thijsse, 1965). The study of such micro-organisms in natural systems has been confined to prospecting for oil fields (Brisbane & Ladd, 1965; Davis, Raymond & Stanley, 1959; Hitzman, 1966), to facilitate the removal of crude oil from its source rock (Dostalek & Spurny. 1958) and the removal of oil where it is a contaminant (Zobell & Prokop, 1966). Geochemical analyses (Smith, 1954; Judson & Murray, 1956; Bergman, 1963; Breger, 1966; Stevenson, 1966) have shown a widespread distribution of hydrocarbons in soils and recent sediments as well as in ancient sediments. By adapting techniques used in the past for petroleum prospecting we have made an ecological survey of the metabolism by micro-organisms of hydrocarbons which occur naturally in an upland moorland soil and underlying shale bands.

The upland moorland site (about 1000 ft above sea level) is situated in the band of Carboniferous Limestone due north of the South Wales coal measures (Grid Reference

SN. 932118). The soil is permanently waterlogged at the sampling points, the water table never falling more than 2 cm. below the soil surface. Since the site is on fenced forestry commission land it is not grazed nor polluted by domestic animals; most of the organic matter entering the system is by turnover of the surface vegetation (*Juncus articulatus* and *Molonia caerulea* are co-dominant) and the washings from a nearby sphagnum pool. The underlying shale band was sampled at the Ogof-Ffynnon Ddu cave system, where the shale was undisturbed and not contaminated by surface soil washings as it is at its point of emergence at the upland moorland site.

METHODS

Sampling. The survey results are those of a sampling programme over two years from 1965 to 1967. The upland moorland soil was sampled at three points, at depths of about 5, 20 and 40 cm. The upper samples were in A_0 horizon and the lower samples in the A_1 horizon. In all instances large samples (about 1 kg.) were taken into sterile polythene bags, and about 15 g. wet wt sample was removed aseptically from the centres of these samples in the laboratory for dilution. Similar aseptic techniques were used for sampling the shale in the cave system.

Microbiological methods. The dilution-plate count method was based on that described by Johnson, Curl, Bond & Fribourg (1960). All dilutions were made into quarter-strength Ringer solution and counts made on a sample dry-wt basis. For each dilution plated, five or ten 0·1 ml. samples were spread with a sterile glass rod on to plates of medium. In all cases a second wipe plate was used. All plating was completed by 3 hr after sampling.

Media. Bacteria and actinomycetes were plated on to modified soil extract agar (Bunt & Rovira, 1955); fungi were estimated on peptone glucose agar with added rose bengal and aureomycin (Martin, 1950; Johnson, 1957). Obligate anaerobes were grown in a H_2 atmosphere in an anaerobic jar on the Tryptone soy+salts agar of Iverson (1966); in some cases they were estimated as numbers of sulphite-reducing units in Iron Sulphite Agar (Oxoid). A basal salts medium was used for estimating hydrocarbon-oxidizing micro-organisms. This contained (g./l. distilled water) K_2HPO_4, 1·0; $MgSO_4.7H_2O$, 0·2; $CaCl_2.2H_2O$, 0·1; NaCl, 0·1; $FeCl_3$, 0·01; NH_4NO_3, 0·5; washed Oxoid Ionagar no. 2, 30. The Ionagar no. 2 was washed 24 times in glass-distilled water and twice in ethanol to remove organic contaminants. Selected hydrocarbons were used as sole carbon sources. Spread dilution plates were made, as described, on the basal mineral salts agar. Sterile filter paper was placed in the lid of the Petri dish and 0·5 ml. hydrocarbon soaked on to this filter paper and the plates then sealed with adhesive tape. In this way the given hydrocarbon was provided in the vapour phase. With all samples, but especially those with a low total bacterial count, this method was found to be more reproducible when the sample dilution was treated in one of the following ways. (*a*) Passed through a membrane filter (0·45 μ mesh), the filter rinsed with 10 ml. sterile quarter-strength Ringer solution, and placed on the basal mineral salts agar. (*b*) Centrifuged at 15,000 rev./min. (27,000 *g*) for 20 min., the deposit washed with sterile quarter-strength Ringer solution, centrifuged down and suspended in a known volume of Ringer solution before plating.

All experiments were done at 25°. Plates were counted at 4, 7, 14 and 21 days according to the particular micro-organisms under study. All media were adjusted to the pH value of the samples taken.

Characterization of the microflora. The initial characterization followed the method of Lochhead & Chase (1943) to establish the nutritional groupings of the microflora of the samples. This method was then extended and an estimate was made of numbers of organisms (expressed as % of the total viable population of each sample) capable of growth with selected hydrocarbons as sole carbon + energy (C + E) source. Growth was recorded after incubation for 7 days.

Following this initial survey estimates were made at each sampling period of the numbers of organisms capable of growth on a variety of single hydrocarbons as sole C + E source. The organisms were grown, as described, on basal mineral salts agar with a hydrocarbon provided in the vapour phase. Sealed control plates without hydrocarbon were also inoculated; growth on these control plates would be due to residual organic matter in the Ionagar no. 2 or organic matter introduced with the inoculum; the number of colonies which developed was subtracted from the numbers obtained on the plates to which hydrocarbon had been added. When a particular species was found to have developed on the control plates then it was discounted on the experimental plates unless there was greater than tenfold stimulation in its growth, in which case it was considered to be of importance in the hydrocarbon metabolism of the sample. All instances of growth of species on hydrocarbons were checked by sub-culture on to a medium containing the test hydrocarbons as sole C + E source.

Determination of microbial activity. All the methods involved measurement of stimulation in respiration of samples when various hydrocarbons were added as extra carbon source. For the initial survey soil and shale samples were incubated in 100 ml. Ehrlenmeyer flasks. Each flask contained a sample (about 0·5 g. wet wt) of the soil or shale under study; basal mineral salts solution, 10 ml. (at pH value of sample); 20% (w/v) KOH solution (0·5 ml.) in suspended Durham tube containing fluted filter paper; hydrocarbon (0·5 ml.) absorbed on 5 g. ignited sand. Control flasks contained no hydrocarbon and thermobarometer flasks no sample. A 1 ml. pipette was attached to each flask and at regular intervals over a period of several days a plug of Brodie manometer fluid was introduced into the pipette. The rate of O_2 uptake was thus measured.

This experiment was repeated in conventional Warburg apparatus with the hydrocarbons which did not create vapour-pressure problems. Results in the Warburg respirometer were less reproducible probably because of the small size of sample introduced. Reports in the literature on the use of dried sieved soil (Stotzky & Norman, 1961) led us to attempt this in the hope of increased reproducibility but such treatment altered considerably the respirometric activity of the samples. The results given here were obtained with soil and shale samples which were treated in a blendor for a few seconds and introduced into the Warburg flask by using a wide-mouthed pipette.

Enhancement of CO_2 production by the samples by adding hydrocarbon was measured by adapting the methods of Stotzky & Mortenson (1957) and Jackson (1958). Samples (100 g. wet wt) were mixed with 300 ml. basal mineral salts solution (adjusted to the pH value of the sample) and 10 ml. hydrocarbon absorbed on ignited sand. Endogenous uptake flasks contained no added hydrocarbon and control flasks contained only basal mineral salts solution. CO_2-free water-saturated air was drawn through the sample flasks; CO_2 evolved was collected in N-NaOH and determined titrimetrically. This method was, however, too cumbersome and too expensive of the natural materials to allow the replication necessary for statistical analysis. The

experiment was therefore scaled down to 5 g. wet wt samples incubated in closed vessels. After the incubation period, the CO_2 produced was collected by boiling it off into NaOH, followed by back titration. All respirometric results were corrected for sample dry weight and total viable microbial count of each sample, and finally expressed as percentage stimulation of respiration; the respiration rate of the endogenous or untreated sample was taken as 100%.

In the population and respirometric analyses the data presented are the means of several tests. Each test involved 3, 5 or 10 replicates according to the analytical procedure used. Differences between mean readings were investigated by the *t*-test. All the results discussed differed significantly at $P = 0{\cdot}05$.

Chemical and physical analyses of samples. Throughout the period of study the soil or shale at the sampling sites were measured for pH value with a portable pH meter (Analytical Instruments Ltd.) with a soil probe attachment. E_h values were measured with a platinum electrode with a calomel reference electrode and corrected to pH 7. To obtain readings for the shale samples, they were mixed with distilled water (sample 1 g., distilled water 2·5 g.). Total organic matter was determined by the Walkley–Black method as adapted by Jackson (1958). Total lipids were extracted for 24 hr in a Soxhlet-type extractor with a chloroform + methanol (2 + 1, v/v) mixture. The hydrocarbon content was determined by column chromatography of this extract on silica gel and alumina. The moisture contents of samples were determined by drying to constant weight at 105°.

RESULTS

Numbers of micro-organisms

Counts of micro-organisms in the soil samples over a period of 1 year showed typical poor soil annual cycle with a major peak in autumn and a minor one in spring. There was a decrease in numbers with depth of sample. This decrease applied to aerobic and anaerobic chemo-organotrophs (Fig. 1). No such seasonal variation was found with the shale samples, due to the more stable cave environment (numbers varied from 3×10^5 to 1×10^6 organisms/g. dry wt shale. Anaerobic chemo-organotrophs were barely detectable in the shale samples. Fungi accounted for about 10% of the colony-forming particles of all samples.

Physical and chemical analyses

The shale was at about pH 7, the soil samples were acid, increasingly with depth (Table 1). Lowest E_h values and maximum organic matter, lipid and hydrocarbon concentrations were found in the 20 cm. deep soil sample. Normal paraffins were found in all samples.

The activity of hydrocarbon-oxidizing organisms

The initial population survey to determine the numbers of hydrocarbon-utilizing organisms in the samples showed the highest proportion of hydrocarbon oxidizers in the 20 cm. soil sample. When the experiment was repeated with solid media with hydrocarbon provided in the vapour phase, the same general pattern was maintained (Table 2).

Respirometric analyses to determine the microbiological activity of the soil samples show maximum stimulation by added hydrocarbon in the 20 cm. sample. This is seen in the O_2 uptake of the samples (Tables 3, 4) as well as in the amount of CO_2 evolved (Table 3). Although there are some inconsistencies (e.g. high activity in the 40 cm. soil samples on addition of *n*-octane) it is clear that 20 cm. soil samples show highest hydrocarbon oxidizing activity, in relation to its total population. High figures obtained with shale samples are dealt with later.

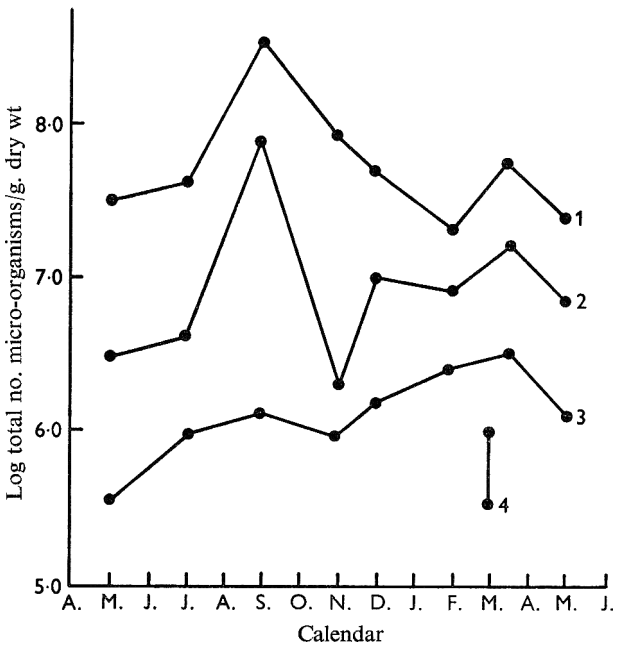

Fig. 1. Seasonal variations in total counts of bacteria and fungi in soil and shale samples. Soil 4 cm. sample, 1; 20 cm. sample, 2; 40 cm. sample, 3; Shale sample, 4. *Experimental conditions*: numbers of bacteria, actinomycetes and fungi were estimated by dilution counts on soil extract agar and peptone dextrose agar. Incubated at 25°. Shale samples showed no seasonal variation and the plots indicate the extremes of variation of numbers along the shale band.

Table 1. *Physical and chemical analysis of samples. Results of a 2-year period of study*

	Soil Sample depths (cm.)			
	5	20	40	Shale
pH	4·8–6·0	3·8–5·0	3·2–3·8	7·2–7·5
E_h (mV.)	+350	+165	+190	+457
Moisture content % dry weight	300–400	550–900	550–900	5–10
Organic matter (mg./g.)	150	800	400	15
Lipid and hydrocarbons (mg./g.)	1–9	5–60	2–20	0·1–1·0

Table 2. *Numbers of organisms capable of growth on selected hydrocarbons*

Sample dilutions were either washed by centrifugation or by membrane filtration. The washed samples or membrane filters were plated on to basal salts agar (set at pH sample). 0·5 ml. hydrocarbon was introduced into the lid of each sealed plate and thus the carbon source was provided in the vapour phase. Control plates contained no hydrocarbon. Incubated for 14 days at 25°. Percentage of total populations of samples capable of growth on hydrocarbons.

	Soil Sample depths (cm.)			
Hydrocarbon	5	20	40	Shale
n-Octane	3 (2–7)	4 (12–25)	1 (4–13)	0·5 (5)
n-Decane	4	8	2	1·5
n-Codecane	13	17	20	3
n-Pentadecane	7	10	10	8
n-Hexadecane	13 (15)	15 (20–30)	8 (12–15)	8 (8–10)
Benzene	*— (0·5)	2 (5)	1 (< 0·5)	1 (2)
Toluene	—	—	—	0·5
Cyclohexane	0·5 (0·5)	3 (6)	0·5 (0·5)	1 (1)

* — Indicates that growth was not detectable by this method.
( ) Indicates figures obtained by adaptation of Lochhead & Chase method (1943).

Table 3. *Stimulation of sample respiration by the addition of hydrocarbons*

5 g. (wet wt) sample was incubated with 0·5 ml. hydrocarbon (adsorbed on to ignited sand) and 10 ml. basal salts solutions (set at pH of sample). A plug of Brodie manometer fluid was introduced into the 1 ml. pipette attached to the reaction vessel. O_2 uptake could thus be followed. Incubated at 25°. Percentage stimulation of O_2 uptake.*

	Soil Sample depths (cm.)			
Hydrocarbon	5	20	40	Shale
n-Octane	300	350	730	2480
n-Decane	510	1500	370	220
n-Dodecane	210	1750	590	115
n-Hexadecane	45	1170	1000	150
Benzene	−160	270	−280	940
Toluene	60	−40	−40	800
Cyclohexane	680	390	40	210

* Rate of respiration of untreated sample is taken as 100 %.

Table 4. *Stimulation of sample respiration by the addition of hydrocarbons*

These experiments were conducted in a conventional Warburg apparatus. Each flask contained, homogenized soil, 2·0 ml.; 20 % (w/v) KOH, 0·2 ml.; quarter-strength Ringer solutions, 0·75 ml.; hydrocarbon 0·05 ml. Control flasks contained no hydrocarbon. Incubated at 25°. Percentage stimulation of O_2 uptake.*

	Soil Sample depths (cm.)			
Hydrocarbon	5	20	40	Shale
n-Decane	60	100	20	200
n-Dodecane	160	400	30	1000

* Rate of respiration of untreated sample is taken as 100 %.

In almost all samples a greater proportion of the population was capable of oxidizing longer chain *n*-aliphatic hydrocarbons than the shorter chain *n*-aliphatics, alicyclic or aromatic hydrocarbons. Comparatively low figures obtained with *n*-pentadecane and *n*-hexadecane are explained by their decreased availability due to lower solubility and volatility at the temperatures used.

We note that maximum hydrocarbon oxidation occurred where organic matter, lipid and hydrocarbon accumulation were at a maximum (Table 1). The dominant microbial genera responsible for hydrocarbon oxidation in the samples studied were noted (Table 6). Several species of microfungi, although comparatively low in numbers, played an important part in the hydrocarbon-oxidizing activity of all the samples.

Table 5. *Stimulation of sample respiration by addition of hydrocarbons*

Samples were incubated in closed flasks. Each flask contained, sample, 5 g. (wet wt); basal salts solution, 10 ml. (set at pH of sample); hydrocarbon, 0·5 ml. (adsorbed on to ignited sand). The samples were incubated at 22° for 3 days and then the gaseous contents were boiled off through 20 ml. 0·1 N-NaOH. CO_2 production was determined titrimetrically. Percentage stimulation of CO_2 production.*

	Soil Sample depths (cm.)			
Hydrocarbon	5	20	40	Shale
n-Octane	25	50	20	140
n-Decane	105	25	30	540
n-Dodecane	210	1100	780	440
n-Pentadecane	360	650	320	220
n-Hexadecane	330	580	50	160
Benzene	−30	−75	−30	−80
Toluene	−5	−100	−30	−140
Cyclohexane	−65	75	−17	−60

* Rate of respiration of untreated sample is taken as 100 %.

DISCUSSION

The media used in this survey were chosen from many tested for maximum reproducibility of results. Adverse weather conditions (e.g. heavy rain) altered the physical, chemical and microbial characteristics of the soil considerably. The shale was free from climatic and seasonal variations.

The sample of lowest E_h values (the 20 cm. soil sample) was where most lipid and hydrocarbon material accumulated, and where the highest proportion of hydrocarbon-metabolizing micro-organisms were detected. The higher hydrocarbon content of this sample has probably caused adaptation of the microbial population resulting in the higher proportion of micro-organisms capable of hydrocarbon metabolism. Total numbers of hydrocarbon-oxidizing micro-organisms may be higher in the surface (5 cm.) soil sample, but other (e.g. physiochemical) factors may limit the development of the populations of the deeper soil samples and shale. Therefore, when comparing the nutritional characteristics of various populations, the numbers of a particular type of micro-organism should be expressed as a function of the whole population.

The presence of local soil microbial populations adapted to hydrocarbons forms the basis of microbial methods of oil prospecting (Brisbane & Ladd, 1965) where gross differences occur between soils overlying oil and gas seeps and those not. The use of a

Table 6. *Dominant microbial genera responsible for hydrocarbon oxidation in the samples*

Hydrocarbon	Soil Sample depths (cm.) 5	20	40	Shale
n-Octane	*Saccharomyces* Unidentified Gram-negative rod *Bacillus* *Pseudomonas* *Mycobacterium* *Arthrobacter*	*Penicillium* *Nocardia* Unidentified Gram-negative rod	*Aspergillus* *Pseudomonas* *Nocardia*	*Saccharomyces* *Micrococcus* *Arthrobacter* 2 (spp.)
n-Dodecane	*Penicillium* (2 spp.) *Cladosporium* (2 spp.) *Botrytis* *Trichoderma* *Spicaria* *Saccharomyces* (3 spp.) Unidentified Gram-negative rod *Pseudomonas*	*Penicillium* (3 spp.) *Saccharomyces* (2 spp.) *Candida* *Bacillus* *Pseudomonas*	*Pseudomonas* *Nocardia* (2 spp.) *Mycobacterium*	*Penicillium* *Corynebacterium* *Micrococcus* (2 spp.)
n-Pentadecane	*Penicillium* (2 spp.) Sterile Basidiomycete (unidentified) *Cephalosporium* *Saccharomyces* *Candida* *Corynebacterium* *Micrococcus* Unidentified Gram-negative rods	*Penicillium* (3 spp.) *Saccharomyces* (2 spp.) *Pseudomonas* *Nocardia*	*Penicillium* (2 spp.) *Aureobasidium*	None isolated
n-Hexadecane	*Penicillium* *Trichoderma* *Saccharomyces* Unidentified member of the Form Order *Mycelia sterilia* *Pseudomonas* *Mycobacterium* *Corynebacterium* *Nocardia* *Bacillus*	*Penicillium* (3 spp.) *Saccharomyces* (2 spp.) *Candida* *Mycobacterium* *Bacillus*	*Penicillium* (3 spp.) *Aspergillus* *Saccharomyces* Unidentified sterile mycelium *Bacillus* *Nocardia* Unidentified gram-negative rods	*Penicillium* *Aspergillus* *Saccharomyces* *Arthrobacter* (3 spp.) *Micrococcus* (4 spp.) *Corynebacterium*
Benzene	None isolated	*Penicillium* Unidentified sterile mycelium	None isolated	None isolated
Cyclohexane	*Penicillium*	*Corynebacterium* *Pseudomonas*	Unidentified sterile mycelium	*Corynebacterium* (2 spp.) *Micrococcus* *Nocardia* *Sarcina*

range of liquid instead of gaseous hydrocarbon substrates, accompanied by suitable statistical analyses allowed us to detect much smaller changes in the microflora of the samples.

All respirometric analyses were made using samples with microbial populations in the *in vivo* state. The conventional Warburg respirometer showed an immediate

stimulation in O_2 uptake on addition of hydrocarbons to the samples, indicating that a section of the sample microflora was already adapted to hydrocarbon metabolism. Respirometric analyses could only be used to compare samples of a similar nature (e.g. similar organic matter content). For example, the shale samples contain low numbers of hydrocarbon oxidizers but the respirometric results indicate a stimulation of as high as 1000% on addition of hydrocarbons. The shale contains much less easily oxidisable organic matter than the soil. Therefore stimulation in its respiration cannot be compared with that in soil because of the much lower rate of endogenous respiration in the shale. Very little is known about the effect of such varying soil factors on the growth of hydrocarbon-utilizing micro-organisms (Brisbane & Ladd, 1965), and differences in rates of endogenous respiration makes it difficult to interpret the stimulation of metabolism in a quantitative manner. Nevertheless, the results do indicate the presence or absence of significant numbers of hydrocarbon-oxidizing micro-organisms.

The use of solid media to demonstrate the presence of organisms capable of oxidizing gaseous hydrocarbons has been reported in the past (Davis *et al.* 1959). We have used this technique and provided the liquid hydrocarbon in the vapour phase, but we found that pretreatment of the samples by membrane filtration or washing made the technique somewhat laborious.

Any one species of hydrocarbon-oxidizing micro-organism can usually metabolize longer chain *n*-aliphatic hydrocarbons more easily than short chain *n*-aliphatic, alicyclic or aromatic hydrocarbons (Johnson, 1964). This survey has shown that this is also a property of naturally occurring mixed populations. In all samples a higher proportion of the population was capable of oxidizing *n*-dodecane, *n*-pentadecane, and *n*-hexadecane and *n*-octane, benzene, toluene or cyclohexane. The microbial genera responsible for hydrocarbon metabolism have been repeatedly and consistently isolated from the samples.

This survey is part of a wider scheme of study concerned with the role of micro-organisms in the biogenesis and turnover of naturally occurring hydrocarbons, and was carried out during the tenure of an S.R.C. Studentship (J.G.J.). We thank the Forestry Commission for access to the upland moorland site.

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Studies on the Regulation of Glucose-metabolizing Enzymes in *Bacillus subtilis*

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SUMMARY

The presence or absence of regulatory control mechanisms has been studied for the enzymes 6-phosphogluconate dehydrogenase, glucose 6-phosphate dehydrogenase and hexokinase in *Bacillus subtilis*. The cellular levels of these enzymes have been investigated during steady exponential growth in various media and following growth shifts. Comparison has been made of the behaviour of these enzymes with that of β-galactosidase in a strain of *Escherichia coli* in which the regulator and operator genes are deleted. Criteria for recognizing truly constitutive enzymes are discussed, and it is tentatively concluded that while the synthesis of the dehydrogenases is controlled, that of hexokinase is probably constitutive.

INTRODUCTION

Nearly forty years ago Karström (1930) distinguished between two classes of enzymes in micro-organisms. Those which were always present regardless of the growth conditions were termed 'constitutive', and although the content of such enzymes within the cells was found to differ in different media, the extents of the variations were small. By contrast, 'adaptive' enzymes were formed in response to a definite factor in the environment, and their concentrations changed very greatly in cells cultured in varying conditions. Subsequent studies have shown that the synthesis of some adaptive enzymes is stimulated as much as 1000-fold by inclusion of appropriate substances in the medium. In other cases a less marked change is observed, amounting perhaps to a tenfold difference.

The years following Karström's publication have witnessed an intensive study of the mechanism of enzyme adaptation, culminating in a number of models which have been proposed to account for the mechanisms controlling the rates of enzyme synthesis. It is clear that the control is, at least in some cases, negative in character (Jacob & Monod, 1961), with specific regulator molecules responding to appropriate small effector molecules in such a way as to prevent the formation of the related enzyme. From some of these controlled systems constitutive mutants have been isolated in which damage to the normal regulatory mechanism has resulted in synthesis of the enzyme independently of the presence or absence of effector molecules. Other types of less specific regulation are recognized which lead to the failure of enzyme synthesis even though the specific inducing effector molecule is present. Many catabolic enzymes are sensitive in this way to an imbalance in the over-all metabolic state of the cell, a condition designated 'catabolite repression' by Magasanik (1961). Catabolite

repression is observed with wild-type cells possessing an intact regulator system, and with mutants in which part of the regulatory system is non-functional (Brown, 1961; Mandelstam, 1962; Moses & Prevost, 1966). In other mutants totally devoid of the regulatory system, part of the catabolite repression response is absent (Palmer & Moses, 1967).

There is some reason to believe that in addition to constitutive strains isolated by artificial selection procedures in the laboratory there also exist in nature wild-type constitutive systems in which the synthesis of certain enzymes might be outside the control of specific regulators. Pardee & Beckwith (1963), in discussing the control of constitutive enzymes, have considered four possible explanations to account for such cases: (i) inducer and (catabolite) repressor are both absent; (ii) inducer is present in constant amount, and repression is absent; (iii) inducer is absent while repression is constantly effective; (iv) induction and repression are always in balance, thus permitting a constant rate of enzyme synthesis irrespective of physiological state. Pardee & Beckwith (1963) briefly discussed a number of enzymes which might truly be constitutive in wild-type strains, but recognized the difficulty of deciding among the above four possible reasons for constitutivity and pointed out the need for further study of these and other examples.

The phenomenon of wild-type constitutivity is of fundamental biological interest. A cell which lacks a means of controlling the synthesis of certain of its components has, compared with its neighbours, an element of inflexibility which sooner or later is likely to prove of selectional importance. Thus, constitutivity based on the absence of both inducing and repressing factors has a biological significance different from constitutivity depending on a balance of these influences, since in the latter case further study might elucidate conditions under which the balance may change and alter the rate of enzyme synthesis.

The problem appeared to us of sufficient interest to warrant further investigation. The glucose-metabolizing enzymes of *Bacillus subtilis* were selected for study because glucose metabolism is usually regarded as being a constitutive function and the enzymes themselves are simple to measure in this organism.

METHODS

Organisms. For most of these studies a prototrophic non-sporogenic strain of *Bacillus subtilis* was used which was obtained from the Department of Bacteriology, University of California, Berkeley. For comparative purposes use has also been made of *Escherichia coli* o_{67}^{c} (from E. Steers) which carries a total deletion of the regulator and operator genes of the *lac* operon (Steers, Craven & Anfinsen, 1965).

Media and growth measurements. Cells were grown at 37° in the following media with stirring: minimal medium 63 (Pardee & Prestridge, 1961) supplemented with 0·2 % (w/v) of the appropriate carbon source; glucose-tris minimal medium containing 0·7 mM-P_i (Moses, 1967); glutamate-minimal medium (Hartwell & Magasanik, 1963); and nutrient broth (Difco). Growth was followed by turbidity; at an extinction of 1·0 in a 1 cm. cuvette at 650 mμ, 1 ml. of bacterial suspension contained 225 μg./ml. of bacterial protein (Moses & Prevost, 1966).

Measurements of enzyme activities. In preliminary studies on the assay techniques for the three glucose enzymes it was found that some procedures for destroying the

cellular permeability barrier inactivated hexokinase but not the dehydrogenases for glucose 6-phosphate and 6-phosphogluconate. For example, treatment with toluene, which is a standard procedure in the assay of β-galactosidase in *Escherichia coli* and histidase in *Bacillus subtilis*, totally inactivated hexokinase. Lysis of *B. subtilis* by lysozyme in the absence of glucose also resulted in a low activity of hexokinase. This was discovered in shift experiments of the type to be reported below. There was a large discontinuous increase in the measured activity of hexokinase in samples of the cultures taken immediately after the addition of glucose to cells growing on another substrate. This was not due to a sudden synthesis of hexokinase, as the following experiment shows.

Cells growing exponentially on nutrient broth were treated with chloramphenicol (100 μg./ml.). Growth measured by turbidity ceased immediately. Sampling was started 15 min. later and was continued for 10 min. Sufficient glucose solution (1 M) was then added to bring the glucose concn. to 10 mM, and sampling was continued for the next 35 min. Figure 1 shows that within 30 sec. of adding glucose the measured activity of hexokinase increased nearly fivefold and remained at that level for the rest of the experiment. Chloramphenicol itself, at concn. up to at least 100 μg./ml., had no effect on hexokinase activity. The activity of hexokinase was apparently partially destroyed by lysis in the absence of glucose, but the presence of the sugar appeared to effect a stabilization. All experiments requiring hexokinase determinations were therefore performed by lysing cells in the presence of glucose.

The three enzymes of glucose metabolism were measured on different portions of the same culture. Samples (0·2 ml.) of the bacterial culture were added to weighed vials previously charged with 0·2 ml. of the following solutions: tris-HCl (0·1 M), pH 7·6, containing lysozyme (0·1 mg./ml.) and EDTA (2·5 mM). Unless the culture medium already contained it, glucose (10 mM) was also added to the lysozyme solution. The vials were reweighed to determine accurately the volume of bacterial culture taken and were allowed to remain at room temperature for about 1 hr. They were then brought to 37° and substrates were added in a volume of 0·6 ml. For 6-phosphogluconate dehydrogenase the substrate solution contained 6-phospho-D-gluconic acid (tricyclohexylammonium salt) (17 mM), $NADP^+$ (1·08 mM) and $MgCl_2$ (1·33 mM) in 0·1 M-tris-HCl, pH 7·6. For measurement of glucose 6-phosphate dehydrogenase the solution contained glucose 6-phosphate (Na_2 salt) (33 mM), $MgCl_2$ (1·33 mM), $NADP^+$ (1·08 mM) and 6-phosphogluconic acid dehydrogenase (0·023 enzyme units/ml.) in 0·1 M-tris-HCl, pH 7·6. For assay of hexokinase activity the solution contained the following substances in 0·1 M-tris-HCl, pH 7·6: ATP (4 mM); $NADP^+$ (1·08 mM); glucose (10 mM); $MgCl_2$ (4mM); glucose 6-phosphate dehydrogenase (0·35 enzyme units/ml.) and 6-phosphogluconic acid dehydrogenase (0·018 enzyme units/ml.). Incubation was allowed to proceed for 25 min. at 37° and the reactions were terminated by adding to each vial 0·4 ml. of 0·75 M-Na_2CO_3. The extinctions were read at 340 mμ with a Cary model 14 spectrophotometer against the appropriate blanks. In each of these reaction mixtures the enzyme under study was the rate-limiting factor; added enzymes were present in excess amounts. Preliminary experiments showed that the rate of NADPH production was almost linear for each of the enzymes for over 30 min. Departures from linearity were not affected by twofold changes in the concentration of any of the reactants except the lysed cells. Since all the samples from a particular experiment were assayed simultaneously for the same length of time they can be considered in a comparative manner even if the true zero time rates were slightly but consistently in error.

β-Galactosidase was also measured in weighed samples of cell suspension as described by Palmer & Moses (1968).

In each case one enzyme unit is defined as that quantity of enzyme catalysing the production of 1 mμmole of measured product/min. at 37°.

Chemicals. Chloramphenicol was a gift from Parke, Davis and Co., Detroit, Michigan, U.S.A.; all other biochemicals and enzymes were from Calbiochem, Los Angeles, California, U.S.A.

RESULTS

Growth experiments. Experiments were performed to determine whether temporary exposure to glucose would facilitate metabolism of the sugar when it was again introduced into the medium. Cells of *Bacillus subtilis* were grown in minimal medium (1 mM-P_i) with one of a number of carbon sources. During the period of exponential

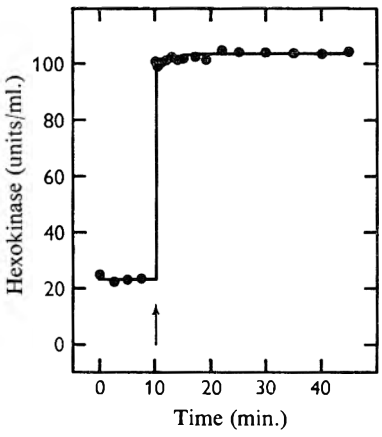

Fig. 1

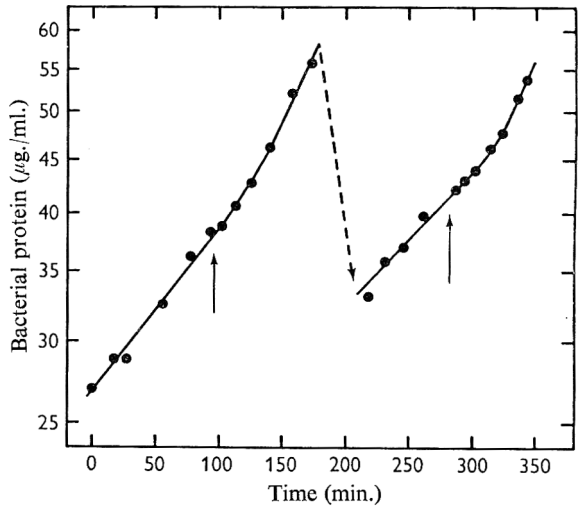

Fig. 2

Fig. 1. Effect on hexokinase activity of the presence and absence of glucose during cell lysis. Cells growing on broth were treated with chloramphenicol (100 μg./ml.). When growth was observed to have ceased, samples were removed at intervals into lysozyme solution without added glucose. Glucose (10 mM) was added to the culture at 10 min. (↑) and sampling was continued. Each sample was later measured for hexokinase activity.

Fig. 2. Growth shifts from alanine to glucose. Cells growing on alanine-minimal medium were supplied with glucose at ↑. After following growth by turbidity for about 80 min. the cells were separated from the medium and resuspended in fresh alanine-minimal medium (dashed arrow). Growth was followed to re-establish the rate on alanine and glucose was again added (↑). The response to glucose was observed for a further 60 min.

growth glucose (10 mM) was added and the growth response observed. With cells cultured on maltose no change in growth rate was obtained on adding glucose. When acetate, alanine, glycerol, proline or succinate were the initial sources of carbon the growth rate increased gradually to a new and higher rate which was reached in 60–70 min. (Fig. 2). After about one doubling in the presence of glucose the cells were filtered through a Millipore membrane filter (0·45 μ pore size), washed, and resuspended in the original medium. Growth was promptly resumed at approximately the

rate characteristic for the original substrate. After a further doubling of the cell mass in the original substrate, glucose (10 mM) was again added. The change in growth rate on addition of this second quantity of glucose was very similar to the first: again a gradual increase in the growth rate was obtained reaching a new maximum rate in about 1 hr.

There is much variability in the growth response of bacteria when glucose is introduced into the medium. In some cases the effect is very rapid: in *Escherichia coli*, for example, a shift from glycerol to glucose is immediate at high (0·1 M) concentrations of P_i, but delayed at lower concentrations (Palmer & Moses, 1967). The present results show that adding glucose for one cell doubling to *Bacillus subtilis* growing on another substrate did not accelerate the second growth response to glucose after the cells had been returned to the original medium for one generation. Thus, specific enzyme synthesis did not appear to be involved in responding to glucose and the delay was more probably metabolic in origin, perhaps entailing a readjustment of the balance of intermediary metabolic pool sizes, etc. By implication, then, the cells were already adequately equipped enzymically to deal with glucose when grown on a variety of other substrates.

Support for this contention has been obtained by H. G. Ungar (unpublished work) in the course of studies on the growth-shift with *Escherichia coli* from acetate to glucose in media containing 5 mM-P_i; this shift results in a slow (60 min.) attainment of the enhanced growth rate. Using the technique described by Prevost & Moses (1967), Ungar studied the release of $^{14}CO_2$ from labelled glucose during the shift and showed that $^{14}CO_2$ evolution began virtually immediately (less than 1 min.) even in the presence of concentrations of chloramphenicol high enough to preclude any possibility of protein synthesis. Thus, glucose may enter metabolism rapidly, yet not result in an immediate increase in the growth rate.

Growth shifts. In an attempt to discover whether glucose in the medium specifically increased the content of the glucose enzymes, experiments were performed in which glucose was added to cells growing on other substrates. Since adding glucose often resulted in an increase in growth rate, other types of growth shift, not involving glucose, were investigated to determine the effect of increasing the growth rate without adding glucose.

Supplying glucose to cells growing exponentially in glutamate-minimal medium invariably resulted in a considerable (50–60 %) reduction in the differential rate of hexokinase synthesis (Fig. 3). The growth rate itself increased gradually over about 1 hr to a rate approximately double that in glutamate. Glucose did not reduce the rate of synthesis for the dehydrogenases for glucose 6-phosphate and 6-phosphogluconate. In some experiments increases in their rates of synthesis were observed, usually after a lag (Fig. 3). In other cases the increases were small enough to be insignificant.

When glucose (10 mM) was added to cells on nutrient broth hexokinase synthesis was not affected and the rates of synthesis of the above two dehydrogenases increased by up to 25 % in a number of experiments (Fig. 4). There was no significant change in the growth rate.

These results suggested the possibility of a specific inducing effect by glucose on the two dehydrogenases. However, the apparent repressive effect of glucose on the rate of hexokinase synthesis in glutamate-grown cells suggested that this might be associated with an increase in growth rate rather than a specific repression of enzyme synthesis.

Since hexokinase is the first enzyme of glucose catabolism one would expect glucose to enhance rather than reduce the rate of its synthesis. The behaviour of these three enzymes was therefore studied in shifts resulting in a marked increase in growth rate, but without using glucose to achieve this.

In one experiment glycerol (22 mM) was added to cells growing exponentially in glutamate-minimal medium. Growth ceased immediately and resumed about 30 min. later at a greatly increased rate (Fig. 5*a*). Glycerol is probably metabolized by an inducible enzyme system (by analogy with the situation in *Escherichia coli* (Hayashi & Lin, 1965)) and the delay before growth started in glycerol may be ascribed to the necessity of inducing the glycerol enzymes. Why the addition of glycerol terminated

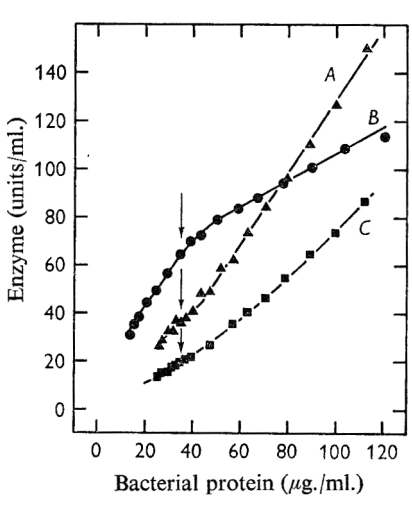

Fig. 3

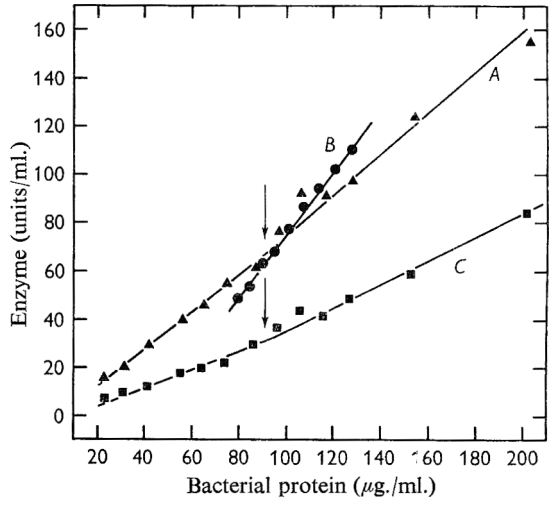

Fig. 4

Fig. 3. Effect on the synthesis of the glucose enzymes of adding glucose to cells in glutamate-minimal medium. Glucose (10 mM) was added at arrows. Growth was measured by turbidity. *A*, glucose 6-phosphate dehydrogenase; *B*, hexokinase; *C*, 6-phosphogluconate dehydrogenase.

Fig. 4. Effect on the synthesis of the glucose enzymes of adding glucose to cells in broth. Glucose (10 mM) was added at arrows. Growth was measured by turbidity. *A*, glucose 6-phosphate dehydrogenase; *B*, hexokinase; *C*, 6-phosphogluconate dehydrogenase.

growth on glutamate is not understood. Shifting from growth on glutamate to growth on glutamate plus glycerol slightly depressed the synthesis of the two dehydrogenases, and reduced the rate of hexokinase synthesis to only 41 % of the earlier rate on glutamate (Fig. 5). Another shift-up without glucose was performed by adding 0·5 ml. of a 50-fold concentrated solution (40 %, w/v) of nutrient broth to 50 ml. of a culture growing in glutamate-minimal medium. The growth rate immediately increased three-fold (Fig. 6). Both dehydrogenases showed a period of decreased synthesis lasting 32–42 min. (about one generation) after which the differential rates of synthesis recovered to 75–87 % of those obtained before the introduction of broth. Hexokinase synthesis, on the other hand, was immediately reduced to 34 % of the differential rate before broth and this did not alter for at least 85 min. or two generations (Fig. 6). The transient reduction in the rates of synthesis of the two dehydrogenases is of particular interest and will be discussed below.

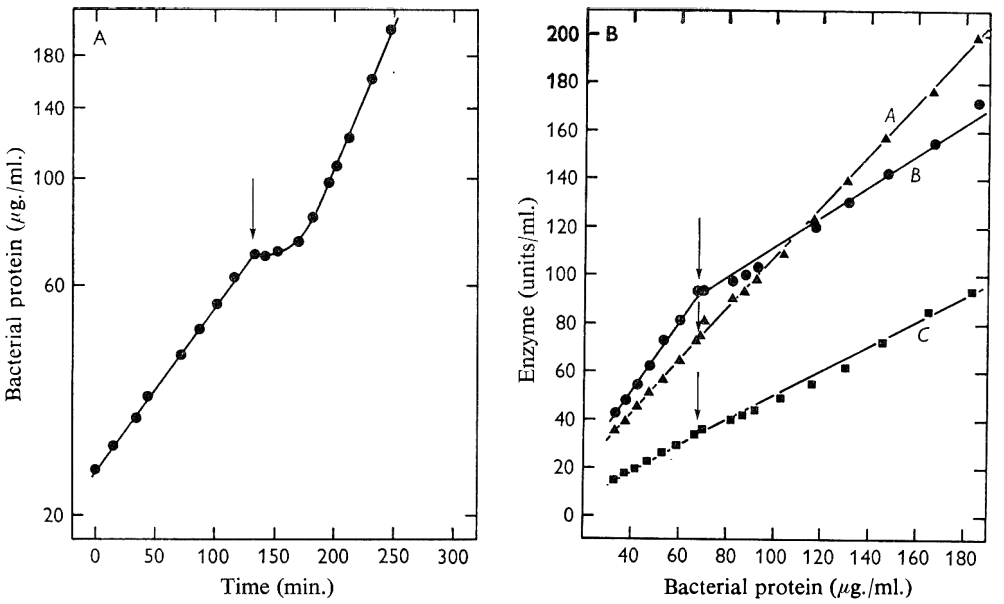

Fig. 5. Effect on growth and on the synthesis of the glucose enzymes of adding glycerol to cells on glutamate-minimal medium. Glycerol (0·2 %, w/v) was added at the arrows. Growth was measured by turbidity. A, growth curve; B, differential synthesis of enzymes: *A*, glucose 6-phosphate dehydrogenase; *B*, hexokinase; *C*, 6-phosphogluconate dehydrogenase.

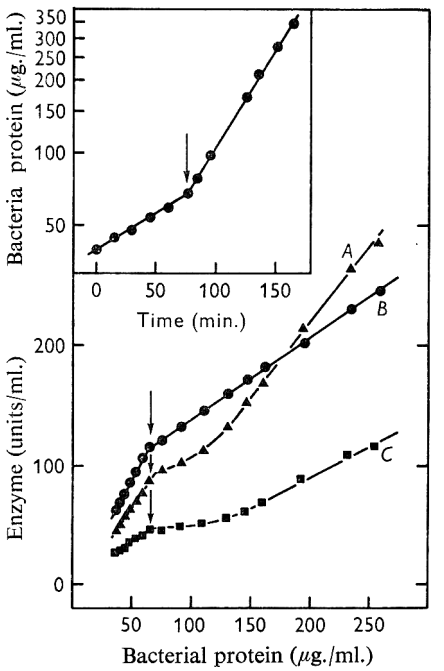

Fig. 6. Effect on growth and on the synthesis of the glucose enzymes of adding broth concentrate to cells on glutamate-minimal medium. Broth concentrate was added at the arrows. Growth was measured by turbidity. Main graph, differential synthesis of enzymes. *A*, glucose 6-phosphate dehydrogenase; *B*, hexokinase; *C*, 6-phosphogluconate dehydrogenase. Inset: growth curve.

Steady-state levels of the three glucose enzymes. A series of measurements were made of the differential rates of synthesis of the glucose enzymes in cells which had been growing exponentially for at least three generations in a number of media. In each case cells were sampled at intervals during the subsequent 60 min. for measurements of enzyme content. Exponential growth at an unchanged rate continued until the end of the sampling period. Table 1 presents the differential rates of synthesis for cells grown on broth, glucose, glutamate, glycerol, proline and succinate. The data in Table 1 demonstrates that the differential rate of hexokinase synthesis is characteristic of the medium rather than the growth rate *per se*. The growth rates in all the media except succinate-minimal were quite similar, yet the rates of hexokinase synthesis varied considerably.

For comparative purposes the differential rates of synthesis of β-galactosidase were studied in *Escherichia coli* o_{67}^{c} growing in the same media (except glutamate, in which it would not grow) (Table 1). This strain has lost the regulator genes (i and o) for the *lac* operon (Steers *et al.* 1965) and the behaviour of β-galactosidase synthesis in growth shifts has been studied by Palmer & Moses (1967). It is therefore of value in determining the pattern of biosynthesis as a function of different growth conditions for an enzyme devoid of all known regulatory mechanisms.

Table 1. *Differential rates of enzyme synthesis in different media*

Cells were grown exponentially for several generations and samples were then removed over a 60 min period while growth was measured by turbidity. Results are expressed as enzyme units/μg. of bacterial protein and as percentages of the rates of synthesis in succinate-minimal medium. Growth rates are recorded as doublings/hr (μ).

	B. subtilis							*E. coli* o_{67}^{c}		
	μ	Glucose 6-phosphate dehydrogenase		6-Phospho-gluconate dehydrogenase		Hexokinase		μ	β-Galactosidase	
Medium		Rate	%	Rate	%	Rate	%		Rate	%
Succinate	0·51	0·944	100	0·653	100	2·863	100	0·89	41·3	100
Proline	0·91	1·075	114	0·693	106	2·049	72	0·28	39·8	96
Glutamate	0·97	1·155	122	0·517	79	1·517	53	—	—	—
Broth	1·04	1·035	110	0·613	94	1·326	46	1·58	27·3	66
Glycerol	1·03	0·974	103	0·653	100	1·205	42	0·80	42·7	100
Glucose	0·98	1·075	114	0·467	72	0·869	30	1·04	16·6	40

DISCUSSION

Are the glucose enzymes constitutive?

Our knowledge of the mechanism of constitutivity in artificial mutants of systems normally inducible or repressible indicates that genetic (or true) constitutivity is the consequence of the absence or malfunctioning of a regulator system. From a practical point of view this makes it very difficult to decide whether a protein whose rate of synthesis varies but slightly under different conditions is constitutive or not. An unvarying rate of synthesis may mean simply that the conditions for varying it have not yet been discovered and not that no such variation is in principle possible.

A decision on constitutivity requires the establishment of criteria. These might be

laid down on a rational basis by deciding, on the grounds of general experience, how a constitutive system would be expected to behave; we must, however, recognize that our general experience may not be sufficient to provide satisfactory criteria. Alternatively, one might compare the enzyme under study with enzymes in artificial mutants which have been analysed genetically and which are known to suffer impairment of the regulatory mechanism. Both approaches will be attempted with the glucose enzymes.

If the glucose enzymes are constitutive we might expect them always to form a constant proportion of the cellular protein, regardless of the culture conditions; if they are inducible it would be reasonable to expect them to be induced either separately by their immediate substrate or by glucose itself if they formed a coordinate induction system. As inducible enzymes they might nevertheless show high basal rates of synthesis in the absence of inducer. Experimental results with glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase showed them both to be present in remarkably constant amounts irrespective of growth conditions (Table 1). While this suggests that these enzymes are constitutive, the fact that in some experiments there were weak responses to the presence of glucose argues that some regulatory control might be possible. The difficulty in making a decision originates from a number of considerations. It is difficult in principle to prove a negative case (the absence of direct regulation), yet evidence for the positive case is not convincing. One can only guess at the identity of a postulated inducer and no progress towards its identification can be made unless clear evidence is obtained that the system is indeed inducible. The gross intracellular pool sizes of likely inducers have not been measured under different growth conditions, but even were this to be done it would have only limited value, since the local concentration of a metabolite at a regulatory site may not of necessity be correlated with its over-all intracellular abundance.

Similar difficulties exist with hexokinase. This enzyme was also found to be present at all times and subject to no more than a threefold variation in activity. Glucose itself would seem to be the most probable inducer, yet its presence in the medium actually decreased hexokinase synthesis and the highest rates of formation were observed when growth took place on substrates far removed metabolically from glucose. We must therefore admit that the behaviour pattern of a naturally constitutive enzyme under varying growth conditions is not readily predictable and it is not possible to decide from the arguments presented above whether or not the three enzymes studied here are under regulatory control.

Arguments based on analogy with a known constitutive system result in a more definite conclusion but it is difficult to assess their validity. For our comparisons we have used the formation of β-galactosidase in *Escherichia coli* strain o^c_{67}. Constitutivity of the *lac* enzymes in *E. coli* is of two types (Jacob & Monod, 1961); regulator mutants (i^-) fail to make a fully functional repressor, while operator constitutives (o^c) fail to respond to normal repressor. Strain o^c_{67} is deleted for both the regulator and operator genes, and is thus fully constitutive on both counts. The formation of β-galactosidase in this strain was studied by Palmer & Moses (1967) and from the response to glycerol to glucose growth shifts and to the presence of certain inhibitors it was concluded that the synthesis of the enzyme might indeed be totally devoid of regulation in this strain. The rates of enzyme synthesis were different in glycerol and glucose but it was suggested that this was due not to a specific type of catabolic repression modulated outside the

lac operon, but rather to a rearrangement of the overall macromolecular composition of the cell under different growth conditions. In that study and in the present one enzyme synthesis is reported on a differential basis, i.e. as a proportion of total protein synthesis. If there is a large change in the synthetic rates for other proteins, with little or no change for the enzyme under study, then the proportion of the whole represented by the particular enzyme (the differential rate of synthesis) will be observed to change.

Table 1 shows that β-galactosidase synthesis in *E. coli* o_{67}^{c} and hexokinase synthesis in *Bacillus subtilis* both show a 2·5–3-fold change in rate in five different media. For both enzymes synthesis is most rapid on succinate and least rapid on glucose. Differences exist between the two systems on the other three substrates, and it is, of course, impossible to compare the two cases too closely, since different organisms are involved. The point is that in both cases variations of similar degrees of magnitude exist in response to growth in a number of media. The dehydrogenases do not show such variability.

Another interesting comparison is found in the kinetics of enzyme synthesis during growth shifts. With wild-type strains of *Escherichia coli*, inducible for the *lac* operon, a transient inhibition of β-galactosidase synthesis is observed during shifts from glycerol, succinate or maltose to glucose (Moses & Prevost, 1966), acetate to glucose (H. G. Ungar, unpublished work), and glycerol to broth (J. Palmer, unpublished work). In strain o_{67}^{c}, on the other hand, no such transient repression of β-galactosidase is observed in glycerol to glucose shifts, although the final rate of enzyme synthesis is lower in glucose than in glycerol (Palmer & Moses, 1967). The behaviour of the two dehydrogenases and hexokinase, reported in the present communication, may also be distinguished in this way. Both dehydrogenases showed marked transient inhibition when the cells were shifted from glutamate into broth (Fig. 6), and a slight effect was also obtained in a shift from glutamate to glucose (Fig. 3); hexokinase showed no transient repression under these conditions, but rather a simple change to a lower rate of synthesis, exactly as observed with *E. coli* o_{67}^{c}.

It seems, then, admitting all the disadvantages of analogy arguments, that a tentative conclusion might be reached that hexokinase synthesis is truly constitutive while the appearance of constitutivity for the dehydrogenases is the result of precise efforts on the part of the cell to regulate the biosynthesis of these two enzymes.

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Size Determination by the Filtration Method of the Reproductive Elements of Group A Streptococcal L-Forms

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SUMMARY

The ability of the L-form of two strains of group A streptococci to pass filters was assessed by several methods. From a comparison of the results, and taking into account the plasticity of the L elements, it is concluded that the size of the reproductive elements of the L-form of the group A streptococcal strains investigated probably lies between 0·45 μ and 0·65 μ. The passage of the elements through filters with pores measuring 0·45 μ or less in diameter may be explained by the ease with which the elements are deformed. The variation between the results obtained with different filtration methods can be attributed to differences in experimental conditions which affect the degree of deformation.

INTRODUCTION

Because filterability is one of the characteristics of the L-forms of bacteria, considerable interest has been shown in the size of these filterable elements. Their capacity to pass filters which retain the bacterial form was first demonstrated by Klieneberger-Nobel (1949) for the L-form of *Streptobacillus moniliformis*, and was later confirmed for the L-forms of other bacteria (Carrère, Roux & Mandin, 1954; Tulasne & Lavillaureix, 1958). Several authors have examined the size of these filterable elements of the L-form of various bacteria by the filtration method (Klieneberger-Nobel, 1949, 1956, 1962; Wittler, 1954; Kellenberger, Liebermeister & Bonifas, 1956; Rada, 1959; Williams, 1963). The results of these experiments give values between 0·175 μ and 0·35 μ for the diameter of the smallest reproductive elements, although Williams (1963) could not demonstrate passage of the L-form of staphylococci through filters with pore size below 0·7 μ.

The ability to grow through filters was investigated for the L-form of Proteus (Silberstein, 1953; Tulasne & Lavillaureix, 1958) and the L-form of staphylococci (Williams, 1963; Molander, Weinberger & Kagan, 1965). The smallest pore sizes through which the staphylococcal L-form grew are reported as 0·05 μ by Molander *et al.* (1965) and 0·7 μ by Williams (1963). The Proteus L-form penetrated filters with pore sizes down to 0·1–0·2 μ (Tulasne & Lavillaureix, 1958), and 0·75 μ–0·50 μ (Silberstein, 1953). The same variation is shown by the results of the filtration experiments performed with the L-form of group A streptococci. The smallest pore sizes of the filters passed in the filtration method were reported as 0·45 μ (Panos, Barkulis & Hayashi, 1960; Mortimer,

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1965) and 0·22 μ (Coussons & Cole, 1968) ,whereas growth through filters has been reported for pore sizes down to 0·10 μ (Dienes & Madoff, 1966; Coussons & Cole, 1968).

A possible explanation for the discrepancy between the results may be differences in the methods used. The ability of the L-form of two strains of group A streptococci to pass filters has therefore been assessed by various methods in a comparative study. Filtration of L-form broth cultures through a series of Millipore membrane filters was done according to the principles laid down by Elford (1938). The capacity for growing through filters was determined in solid and liquid media. Another source of differences, as pointed out by Roux (1960) and Weibull & Lundin (1962), may be the plasticity of the L elements, which makes the filtration method liable to give variable results when applied to the size determination of these deformable particles. The results obtained in the present work support this view.

METHODS

Organisms and cultivation. Two strains of group A, β-haemolytic streptococci were used. Both strains had originally been obtained from Dr L. Dienes (Boston, Mass., U.S.A.). They belonged to the serological types 19 and 12, and were designated GL-8 and AED, respectively. The L-forms had been derived from the parent strains by the penicillin gradient technique according to Sharp (1954). The L-forms were cultivated in a medium composed of 2·8 % (w/v) Brucella Broth (Albimi Laboratories, Inc., Brooklyn 2, New York), sodium chloride 0·56 M and sodium penicillin to 1000 i.u./ml. The medium was solidified with 1 % (w/v) agar (Special Agar Noble, Difco Laboratories, Inc., Detroit, U.S.A.). The L-forms of both strains had been subcultured in brucella broth medium over 100–150 consecutive transfers. The cultures were incubated at 37°. Viable counts, expressed as the number of colony-forming units (c.f.u.)/ml., were determined by the pour-plate technique. The streptococci were grown in Todd-Hewitt Broth (Difco). Nutrient Broth (Difco) was used for the cultivation of the *Serratia marcescens*.

Influenza A virus was grown on fertile hen's eggs, harvested, and purified by adsorbtion and elution from erythrocytes. The titre of the saline suspension was determined by the haemagglutination method.

Filter and filter apparatus. Standard Millipore filter membranes (Millipore Filter Corporation, Bedford, Mass., U.S.A.) of the following types and mean pore sizes were used: SM (5·0 μ), SS (3·0 μ), RA (1·2 μ), AA (0·8 μ), DA (0·65 μ), HA (0·45 μ), PH (0·30 μ), GS (0·22 μ), VC (0·10 μ), VM (0·05 μ) and VF (0·01 μ).

Two types of filter holder were used. (i) The Swinny Hypodermic Adapter (Millipore Cat. No. XX30 012 00) with filters of diam. 13 mm., filtration surface 0·8 cm.^{2}. (ii) The Stainless Pressure Filter Holder (Millipore Cat. No. XX40 047 00), with filters 47 mm. diam., filtration surface 11·3 cm.^{2}. The volumes filtered through the two types of filter holder were 10–15 ml. and 80–100 ml., respectively. The filters, sterilized when necessary with ethylene oxide, and the autoclaved filter holders were assembled aseptically. The Swinny adapter was attached on one side to a sterile 10 ml. standard hypodermic syringe; the other side of the filter holder was provided with a sterile needle to collect the filtrate. After removal of the plunger, the sample was pipetted into the syringe and the entire assembly was fitted by means of rubber rings into a cylindrical glass bell.

The glass bell was then connected by rubber tubing to a pressure vessel. Six glass bells could be connected to the pressure vessel, permitting six samples to be run simultaneously under identical experimental conditions. The pressure, supplied by a pressure pump, was regulated and controlled by the stopcocks and manometer of the pressure vessel. The stainless pressure filter holder was connected to a nitrogen cylinder, the pressure being regulated by the reducing valve.

Filtration. The experiments were done at room temperature (20–22°), under a positive pressure of 0·2–0·3 kg./cm.^{2}. A few experiments performed under negative pressure gave essentially the same results.

Homogeneous suspensions of L-form cultures were prepared by vigorous shaking and subsequent centrifugation at 1000 *g* for 20 min. at 4°. The resulting supernatant fluid, containing about 10^6 to 10^7 colony forming units per ml. (c.f.u./ml.), to be further referred to as 'L-form suspension', was used in the experiments. At the start of each experiment the number of c.f.u./ml. of the suspensions was assayed. Portions of the suspensions held at room temperature during the experiments showed no appreciable change in the number of c.f.u. Immediately after collection, the number of c.f.u./ml. was determined in the filtrates, which were collected either totally or in a number of fractions. In the latter case the fraction containing the highest number of c.f.u./ml. (the maximum concentration) was regarded as representative of the filtration through that filter. Further details are given under Results.

The *Serratia marcescens* cultures, incubated for 4–6 hr, were washed and resuspended in the brucella broth medium. Before suspension in the same medium, the streptococcal cultures were treated 5 times for periods of 1 min. in a ultrasonic disintegrator (MSE, Model 60W, 60 kcyc./sec.) to disrupt the chains.

Growth through filters. Circular pieces of Millipore membrane filters of diam. 0·7 cm. were placed on agar plates and the filter surfaces inoculated with a drop of L-form culture. The plates were then sealed with paraffin, incubated for 7 days, and the filters then removed and the plates inspected for colonies. Control experiments were made with the streptococcal strains GL-8 and AED. For the experiments in liquid medium, the Bellco Parabiotic Chamber (Bellco Glass Inc., Vineland, New Jersey, U.S.A.) was used, filters of various porosities being placed between the compartments. Both compartments were filled with the broth, and one side was inoculated with L-form growth. The uninoculated side was subcultured daily during a 3-day incubation period. Experiments with *Serratia marcescens* and the streptococci GL-8 and AED served as controls.

RESULTS

Filtration experiments

To establish the relationship between filter pore size and particle diameter, the Millipore membrane filters were tested with suspensions of *Serratia marcescens* (size 0·5 $\mu \times 0{\cdot}5$ to 1·0 μ, *Bergey's Manual*, 1957) and influenza A virus (size 0·08–0·1 μ; *Topley & Wilson's Principles*, 1964). The 10 ml. samples of *S. marcescens* suspensions in brucella salt medium were filtered simultaneously through filters of various pore size, employing the Swinny adapter filter holders. The filtrates were collected in portions of 2·5 ml. The number of c.f.u./ml. of the fraction with the highest concentration was recorded. Influenza A virus was suspended in saline, samples (10 ml.) were filtered and collected. With the exception of the saline suspending medium, the experi-

mental conditions were the same as used in the experiments with the L-form suspensions. The results are given in Tables 1 and 2.

For both *Serratia marcescens* and the influenza A virus, a close relationship was found between the pore diameter of the filter membrane just able to retain completely all the dispersed particles, the limiting pore diameter (l.p.d.), and the smallest size of these particles, indicating a direct relation between the l.p.d. and the size of the particles. This was confirmed by the results obtained with the GL-8 and AED strains of group A streptococci (size 0·6–1·0 μ, *Bergey's Manual*, 1957). For both strains an l.p.d. of 0·65 μ was found.

Table 1. *Filtration of Serratia marcescens through Millipore membrane filters*

Samples (10 ml.) of 4 hr cultures resuspended in brucella broth salt medium, were filtered simultaneously through membrane filters of the indicated pore sizes. Filtrates were collected in 4 × 2·5 ml. fractions. The number of c.f.u.*/ml. of the fraction containing the maximum concentration is recorded as log. no. c.f.u./ml. and, in parentheses, as % of the original number (the maximum relative concentration).

	Expt. 1	Expt. 2	Expt. 3	Expt. 4	
	Log c.f.u./ml.		original suspension		
	7·30	5·95	6·95	5·90	Average maximum relative concentration
Filter pore size (mμ)	Filtrates (log c.f.u./ml.)				
3000	5·48 (1·5)	n.t.†	4·48 (0·3)	n.t.	0·9
1200	4·48 (0·2)	3·30 (0·2)	3·30 (0·2)	3·77 (0·8)	0·3
800	4·30 (0·2)	n.t.	3·84 (0·08)	3·69 (0·6)	0·2
650	n.t.	2·30 (0·02)	3·00 (0·01)	1·95 (0·01)	0·01
450	0 (0)	0 (0)	0 (0)	0 (0)	0

* c.f.u. = colony forming unit. † n.t. = not tested.

Table 2. *Filtration of influenza A virus through Millipore membrane filters*

Samples (10 ml.) of saline suspensions were filtered through membrane filters of the indicated pore sizes. Virus titre assayed by the haemagglutination (h.a.) method

	Expt. 1	Expt. 2
	h.a. titre of the original suspension	
	512	64
Filter pore size (mμ)	h.a. titre of the filtrates	
3000	n.t.*	64
220	256	64
100	4	< 4
50	< 4	< 4
10	< 4	< 4

* n.t. = not tested.

Simultaneous filtration of L-form suspensions. The L-form suspensions were prepared from 18–24 hr cultures. In each experiment several 10 ml. samples of one L-form suspension were passed simultaneously through filter membranes of different porosities in Swinny adapter filter holders, and the filtrates collected in 2·5 ml. fractions. Table 3

shows the number of c.f.u./ml. contained in the fraction with the maximum concentration. These data indicate an l.p.d. of 0·30 μ for the L elements.

Sequential filtration of L form suspensions. To rule out the possibility that obstruction of the filter pores could result in too high value of the l.p.d., a further series of experiments was made. L-form suspensions were prepared from 18–24 hr. cultures, and one sample of each suspension was filtered successively through filters with progressively

Table 3. *Simultaneous filtration through Millipore membrane filters of the L-forms of 2 strains of group A streptococci*

Samples (10 ml.) of L-form suspensions prepared from 18 to 24 hr L-form broth cultures, were filtered simultaneously through membrane filters of the indicated pore sizes. Filtrates were collected in 4 × 2·5 ml. fractions. The number of c.f.u.*/ml. of the fraction containing the maximal concentration is recorded as log. no. c.f.u./ml. and, in parentheses, as % of the original number (maximum relative concentration).

	Strain of streptococcus				
	GL-8	GL-8	GL-8	AED	
	Log c.f.u./ml. original suspension				
Filter pore size (mμ)	6·00	6·00	7·30	6·00	Average maximum relative concentration (%)
	Filtrates (log c.f.u./ml.)				
5000	n.t.†	4·95 (9)	6·30 (10)	5·00 (10)	10
800	n.t.	5·30 (18)	6·00 (7)	5·00 (10)	12
650	5·20 (16)	n.t.	6·44 (14)	4·84 (7)	12
450	2·00 (0·05)	1·47 (0·003)	0 (0)	1·00 (0·01)	0·02
300	n.t.	0 (0)	0 (0)	0 (0)	0
220	0 (0)	0 (0)	0 (0)	0 (0)	0

* c.f.u. = colony forming unit. † n.t. = not tested.

Table 4. *Sequential filtration through Millipore membrane filters of the L-forms of 2 strains of group A streptococci*

Samples (10 ml.) of L-form suspensions prepared from 18 to 24 hr L-form broth cultures, were filtered successively through membrane filters of decreasing porosity. After each passage, 1 ml. of the filtrate was inoculated into broth. The absence (−) or the occurrence (+) of growth after 5-day incubation is indicated.

	Strain GL-8			Strain AED		
	Growth obtained from the original suspensions					
	+	+	+	+	+	+
Filter pore size (mμ)	Growth obtained from the filtrates					
1200	+	n.t.*	+	n.t.	n.t.	+
800	+	+	n.t.	+	+	n.t.
650	+	+	+	+	n.t.	+
450	+	+	+	+	+	+
300	n.t.	−	−	−	+	−
220	−	−	−	−	−	−

* n.t. = not tested.

Table 5. *The influence of culture age on the filterability through Millipore membrane filters of the L-forms of 2 strains of group A streptococci*

Samples (80–100 ml.) of L-form suspensions prepared from 6, 12 and 24 hr L-form broth cultures, were filtered through membrane filters with 0·45 and 0·30 μ pore size, and 5 to 7 fractions of equal volume were collected. The results are expressed as log. no. c.f.u./ml., and, in parentheses, as percentage of the original concentration (relative concentration).

L-form of strain	Age of culture (hr)	Mean pore size of filter (mμ)	Log. no. c.f.u./ml. orig. susp.	Log. c.f.u./ml. and relative concentration (%) of filtrate fractions						
				1	2	3	4	5	6	7
GL-8	6	450	6·48	0 (0)	2·30 (0·003)	3·90 (0·1)	4·30 (0·1)	4·47 (0·4)	4·47 (0·4)	4·60 (0·6)
		300	6·48	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	12	450	6·90	0 (0)	2·30 (0·003)	3·47 (0·04)	3·84 (0·1)	3·77 (0·07)	3·30 (0·03)	2·47 (0·004)
		300	6·90	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	24	450	7·30	0 (0)	1·00 (0·0002)	2·84 (0·004)	2·95 (0·005)	3·30 (0·01)	—	—
		300	7·30	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	—	—
AED	6	450	4·84	0 (0)	0 (0)	0 (0)	1·00 (0·002)	1·47 (0·004)	1·47 (0·004)	1·47 (0·004)
		300	6·30	0 (0)	0 (0)	0 (0)	1·00 (0·0005)	1·30 (0·0001)	1·30 (0·0001)	—
	12	450	7·00	0 (0)	3·69 (0·05)	4·90 (0·08)	5·00 (1·0)	5·00 (1·0)	4·90 (0·8)	4·60 (0·8)
		300	7·00	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	24	450	7·00	0 (0)	0 (0)	2·30 (0·002)	2·69 (0·005)	2·69 (0·005)	2·69 (0·005)	—
		300	7·00	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	—

smaller pore size. The experiments were started with 10–15 ml. samples, and after each passage 1 ml. of the filtrate was inoculated into broth. These cultures were incubated for 5 days, and growth assessed from daily subcultures on agar plates. As can be seen from Table 4, essentially the same results were obtained. With the exception of one experiment, the 0·30 μ filtrate remained negative.

Culture age and filterability of L-form suspensions. To assess the influence of the age of the culture on filterability, especially through the limiting filter, L-form suspensions were prepared from cultures incubated for 6, 12, and 24 hr. To study the effects of absorption and clogging on the filtration process, volumes of 80–100 ml. were filtered through 0·30 μ and 0·45 μ filters in stainless pressure filter holders. For the 6 or 7 filtrate fractions of equal volume thus obtained, the number of c.f.u./ml. was determined separately. The results (Table 5) showed that although the L-form suspensions prepared from cultures incubated for 6 and 12 hr seemed to pass a little more readily, the age of the culture did not influence the filtration results with respect to the size of the limiting pore diameter. Except in one experiment (see Table 5) in which a small percentage of the reproductive elements passed it, the 0·30 μ filter retained the L elements.

The first filtrate fractions of the 0·45 μ filter were always negative, the following two fractions gave an increasing number of c.f.u./ml. The maximum number of c.f.u./ml. was found in fractions no. 4 or 5, and remained nearly constant in the following fractions. This result indicates a slow and difficult passage of the reproductive elements through the filter pores. An appreciable effect of clogging of the filter pores, which would have been shown by a decrease of the number c.f.u./ml., was not observed, however.

Growth through membrane filters

Experiments on agar plates. The L-forms of the two streptococcal strains inoculated on Millipore membrane filters of various pore sizes placed on agar plates, grew through filters with pore sizes of 0·22 μ or larger. The passage through the filters was shown by the development of colonies underneath the filters (Pl. 1). Although growth occurred on top of the 0·10 μ filter, penetration of this filter was not observed during the incubation period. The control experiments with the GL-8 and AED streptococcal parent strains showed that, although abundant growth occurred on top of the filters, the bacterial form did not grow through any of the filters.

Experiments in broth medium. The L-form of both streptococcal strains grew through the filter of 0·30 μ pore size. Irregular results were obtained with the 0·22 μ filter.

The *Serratia marcescens* and the streptococcal parent strains used in the control experiments passed through the 0·45 μ and the 0·65 μ filters, respectively. The *S. marcescens* was completely retained by the 0·30 μ and the streptococcus by the 0·45 μ filter.

DISCUSSION

Lederberg & St Clair (1958), Roux (1960), and Weibull & Lundin (1962) have pointed out that the plasticity of the L-form elements may be a factor which determines the filterability of the bacterial L-forms. The deformability of the L elements should therefore be taken into account in the interpretation of the results of filtration experiments. In general, the results obtained in the present study with the L-forms of the GL-8 and AED strain of group A streptococci are in agreement with the results reported in the literature (Panos, Barkulis & Hayashi, 1960; Mortimer, 1965; Dienes & Madoff, 1966;

Coussons & Cole, 1968). However, the question of the significance of the filterability with respect to the size of the filterable and reproductive elements remains.

In these experiments the percentage of reproductive elements passing the filter with a pore size of 0·45 μ varied between 1 % and 0·001 % or even less. A low filtration recovery after filtration of bacterial L-forms through filters with pore sizes of 0·6 μ or smaller has been reported (Klieneberger-Nobel, 1949, 1962; Rada, 1959; Kellenberger *et al.* 1956; Panos *et al.* 1960). The low filtration recovery cannot be solely explained by mechanical obstruction of the filter pores and adsorption on to the filter surface. The similar results obtained in the serial and sequential filtration experiments, and the absence of a decrease in the number of viable elements passing a single filter during filtration indicated that, under the experimental conditions used, mechanical blocking of the filter pores did not influence the results to a significant degree. In both the control experiments and the experiments performed with the streptococcal L-forms, on the other hand, a strong adsorption of elements on to the filters occurred. This is demonstrated by the reduction in viable count of the suspensions after passage through filters with pore sizes many times larger than the diameter of the suspended particles. This retention, which is due to electrostatic charges (see *Millipore Application Data Manual*, 1963, Millipore Filtration Corp., Bedford, Mass. U.S.A.), did not however, influence the relationship between the particle diameter and the size of the limiting pore diameter, as indicated by the control experiments. Furthermore, the number of reproductive L-form elements passing the filters remained nearly constant up to the 0·65 μ filter, but showed a sharp decrease at the 0·45 μ filter. The filtration recoveries obtained with the control suspensions of *Serratia marcescens* and influenza A virus, to the contrary, gradually decreased with decreasing filter pore size. This suggests that either the L-forms constitute a homogeneous population of L elements with respect to size or that passage is effected by means of deformation. In view of the pleomorphic character of the bacterial L-form, the latter explanation seems the more likely. The maximal degree of deformation which is still compatible with viability occurs with filters of 0·45 μ porosity.

The low filtration recovery obtained with this filter can be explained by the assumption that only a few elements are capable to pass these filters. In addition, the results show that the filterability of the streptococcal L-forms was not influenced by the age of the L-form cultures.

Penetration of the 0·30 μ and 0·22 μ filters occurred in the experiments in which the streptococcal L-forms grew through the filters. These filters were not passed by reproductive elements in the filtration experiments. The divergence between the results of the two methods, which is also found in the literature, is perhaps also best explained by the assumption that the pliable L-form elements are able to adjust to and pass through these smaller filter pores by deformation. Apparently, a higher degree of deformation of the L-form elements can be achieved in the slow process of penetration by growth than under the conditions prevailing in the filtration experiments.

The present results suggest that the plasticity of the streptococcal L-form elements is indeed a factor determining the filterability of the L-form. Consequently, a conclusion about the size of the smallest reproductive elements is difficult to reach. The principles governing size determination by the filtration method, i.e. the relationship between limiting pore diameter and particle size (Elford, 1938), are based on the concept of a rigid particle. These principles should therefore not be applied to the results obtained with L-form elements because of their pliability.

The sharp decrease in the number of reproductive elements passing the 0·45 μ filter as compared to the 0·65 μ filter indicates, however, that in all likelihood the size of the smallest reproductive elements of the L-form of the two group A streptococcal strains used in this study lies between these values.

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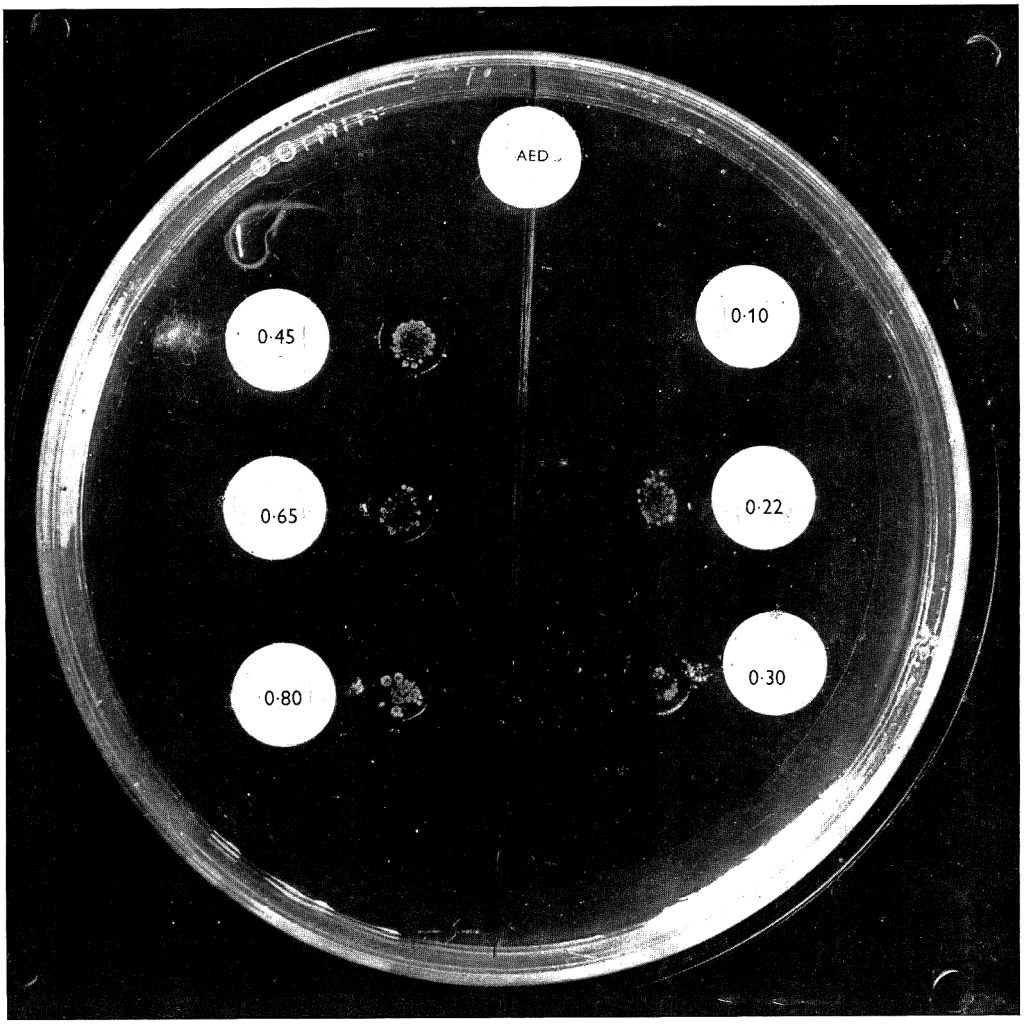
EXPLANATION OF PLATE

Millipore membrane filter discs were placed on the surface of an agar plate and inoculated on top with the liquid growth of the L-form of the AED strain of group A streptococci. The photograph was taken after 5-days incubation, after removal of the filter discs. Penetration of the filters is shown by the development of L-form colonies in the agar underneath the filters. The numbered labels indicate the pore size (μ) of the filters employed. × 1·3.


AED
0·45
0·10
0·65
0·22
0·80
0·30

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Size Determination by Phase-contrast Microscopy of the Reproductive Elements of Group A Streptococcal L-Forms

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SUMMARY

Slide cultures consisting of small agar blocks were inoculated with the L-forms of two group A streptococci strains GL-8 and AED grown in broth. The size of the L-form elements was measured on photographs taken immediately after inoculation of the slide cultures. The diameter of the elements varied between 0·2 and 3·0 μ. The frequency distribution of the diameters has a bimodal form. This bimodality probably represents the size distribution of two classes of L-form elements which can arbitrarily be divided at the diameter of 0·7 μ.

The reproductive elements of the streptococcal L-forms were found, with a few exceptions, to have a minimal diameter of 0·7 μ (average diameter 1·4 and 1·2 μ, respectively; standard deviation 0·3 μ).

Slide culture experiments with concentrated filtrates of 0·45 filters showed that the reproductive elements present in the filtrates ranged in size from 0·8 to 1·7 μ with an average diameter of 1·2 μ (standard deviation 0·2 μ). The filterability of the streptococcal L-form might therefore be mainly due to the plasticity of the L-form elements.

INTRODUCTION

The occurrence of elements of widely differing sizes, with diameters ranging from the resolving power of the light microscope to several μ, is characteristic for the L-forms of bacteria. With respect to the size of the elements capable of reproduction, the results of filtration experiments generally indicate that elements with a diameter of 0·35 μ or even smaller are capable of reproduction (see van Boven, Ensering & Hijmans, 1968). However, studies of the reproductive L-form elements by phase-contrast microscopy (Weibull & Lundin, 1962; Weibull, 1963; Taubeneck, 1962), with the micromanipulator (Roux, 1960) and in stained agar preparations (Dienes, 1967, 1968) have yielded values of 0·6–0·7 μ or larger.

One of the factors considered to affect the passage of L-form elements through filter pores is their plasticity (van Boven *et al.* 1968). This may explain the differences between the results of filtration experiments and direct microscopic measurement; the size of the smallest reproductive L-form elements obtained by the filtration method tends to be too low.

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The filtration experiments with the L-forms of two group A streptococci indicated that the size of the smallest reproductive elements varied between 0·45 and 0·65 μ (van Boven *et al.* 1968). The present paper reports the results of microscopical size determinations of the L-form elements of the same two strains. The size distribution of the elements and the relation between size and reproductive capacity were determined by phase-contrast microscopy of slide cultures. To test the assumption that their plasticity allows L-form elements to pass membrane filters with pores smaller than these elements, the concentrated filtrates from 0·45 μ Millipore membrane filters were studied by the same method. The minimal size of the L-form elements of the two strains of group A streptococci which commenced growth and developed into micro-colonies was 0·7 μ or larger. The L-form elements responsible for the development of growth in the 0·45 μ filtrates had diameters of the same order of magnitude, i.e. 0·8 μ or larger.

METHODS

Organisms and cultivation. The L-forms of the GL-8 and AED strains of group A streptococci and the methods of cultivation were described in the preceding paper (van Boven *et al.* 1968). For the slide cultures, the medium was supplemented with 10% (v/v) inactivated horse serum.

Preparation of the slide cultures. Small agar blocks (7 × 7 mm.) were cut out of 1 mm. thick agar plates and placed on conventional glass slides (76 × 26 mm.). Inoculation was made either by streaking 0·2 ml. broth culture on the agar plate before cutting out the blocks or by placing a loopful of broth culture on the agar block. Coverslips (22 × 22 mm.) were placed on the agar blocks, and the preparation was sealed off with a mixture of petroleum jelly (10%) and paraffin. For counting and orientation in the slide cultures according to Taubeneck (1959), a Formvar film provided with a counting grid (3 × 3 mm.; ruled in 60 × 60 squares of 50 × 50 μ) was attached to each coverslip before use. The slide cultures were incubated at 37°.

Phase-contrast microscopy. A Leitz Ortholux microscope (Ernst Leitz GMBH, Wetzlar, Germany) equipped with the Leitz phase-contrast system was used with the × 63 dry objective (N.A. 0·70) or the × 90 oil immersion objective (N.A. 1·15). Photographs were taken with either a micrographic camera or an automatic microscope camera (Leitz Orthomat Automatic Camera) with a green filter and Ilford Pan F film (Ilford Ltd, Ilford, Essex, England). Negatives were printed and enlarged to give a final magnification of 3600 (× 90 objective) or 2625 (× 63 objective).

Determination of particle size and reproductive capacity. The diameter of sharply-focused single elements was measured on the photographic prints. For non-spherical elements the smallest diameter was taken. The measurements were done in mm. to the nearest 0·1 mm., and converted into μ. To assess the accuracy of the method, the diameter of polysterene spheres with a known diameter of 0·81 μ (Difco Bacto-Latex, 0·81, Difco Laboratories, Detroit, Michigan, U.S.A.) were measured. The average diameter and standard deviation derived from measurements of 30 and 82 polysterene spheres with a × 63 and × 90 objectives, respectively, were 0·8 $\mu \pm$ 0·09 μ and 0·9 $\mu \pm$ 0·04 μ.

The reproductive capacity of the elements was assessed from measurements done in photographs of the same fields made directly after inoculation and at regular intervals during incubation. Comparison of the diameters of the elements in the

successive photographs showed which of the elements had developed into a micro-colony.

Size of filterable elements. Standard Millipore filter membranes of type HA, pore size 0·45 μ, were employed. L-form suspensions were prepared and filtered as previously described (van Boven *et al.* 1968). The filtrates were concentrated by centrifugation at 50,000*g* for 60 min. in a Spinco Model L preparative ultracentrifuge equipped with a no. 30 rotor. Slide cultures were inoculated with the sediment.

RESULTS

Microscopic appearance and particle-size distribution

To study the microscopical aspect of the elements in L-form broth cultures of group A streptococci, slide cultures were prepared from cultures incubated for 15 hr at 37°. Plate 1, fig. 1, 2 show the L-form elements of the GL-8 and AED strain on slide

Table 1. *Size and relation between size and reproductive capacity of the L-form elements of the GL-8 and AED strain of group A streptococci*

Series I. Slide cultures were inoculated from 15-hr L-form broth cultures and photographed immediately afterwards, using a phase contrast microscope with a ×90 oil immersion objective. Particle diameters were measured on the enlarged photographic prints: final magnification ×3600.

Series II. L-form broth cultures incubated for 9 hr were used to inoculate the slide cultures. Photographs were taken immediately after inoculation and at regular intervals during incubation. A ×90 oil immersion phase-contrast objective and a ×63 dry phase-contrast objective were used in the experiments with the GL-8 and AED strains, respectively. The photographs were enlarged to a final magnification of ×3600 and ×2625 respectively.

	Experimental series I Strain				Experimental series II Strain							
	GL-8		AED		GL-8				AED			
	Elements per class				Elements per class							
					Total		Reproductive		Total		Reproductive	
Class (μ)	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
0·2–0·3	158	22·8	308	21·3	15	4·4	0	0	—	—	—	—
0·3–0·4	131	18·9	317	21·9	27	7·8	0	0	6	1·0	0	0
0·4–0·5	64	9·2	169	11·7	30	8·7	0	0	28	4·7	1	0·2
0·5–0·6	33	4·8	100	6·9	19	5·5	0	0	35	5·9	7	1·2
0·6–0·7	19	2·7	93	6·4	15	4·4	0	0	20	3·4	5	0·8
0·7–0·8	23	3·3	50	3·5	8	2·3	2	0·6	51	8·6	27	4·6
0·8–0·9	38	5·5	82	5·7	3	0·9	1	0·3	30	5·1	17	2·9
0·9–1·0	67	9·7	58	4·0	6	1·7	3	0·9	77	13·0	58	9·8
1·0–1·1	37	5·3	59	4·1	11	3·2	11	3·2	54	9·1	43	7·3
1·1–1·2	48	6·9	68	4·7	28	8·1	27	7·8	87	14·7	67	11·3
1·2–1·3	21	3·0	45	3·1	44	12·8	43	12·5	53	9·0	45	7·6
1·3–1·4	24	3·5	39	2·7	44	12·8	43	12·5	42	7·1	40	6·8
1·4–1·5	13	1·9	23	1·6	30	8·7	29	8·4	28	4·7	27	4·6
1·5–1·6	8	1·2	11	0·8	23	6·7	23	6·7	34	5·7	32	5·4
1·6–1·7	3	0·4	11	0·8	18	5·2	18	5·2	19	3·2	17	2·9
1·7–1·8	2	0·3	4	0·3	3	0·9	3	0·9	12	2·0	12	2·0
1·8–1·9	1	0·1	5	0·3	6	1·7	6	1·7	6	1·0	6	1·0
⩾ 1·9	2	0·3	3	0·2	14	4·1	14	4·1	8	1·3	5	0·8
Total	692	100·0	1445	100·0	344	100·0	223	64·8	590	100·0	409	69·1

cultures photographed immediately after inoculation. For comparison, Pl. 2, fig. 5 shows the appearance of a slide inoculated with sterile broth. The photographs demonstrate the pleiomorphic aspect of the L-form elements, especially with regard to size.

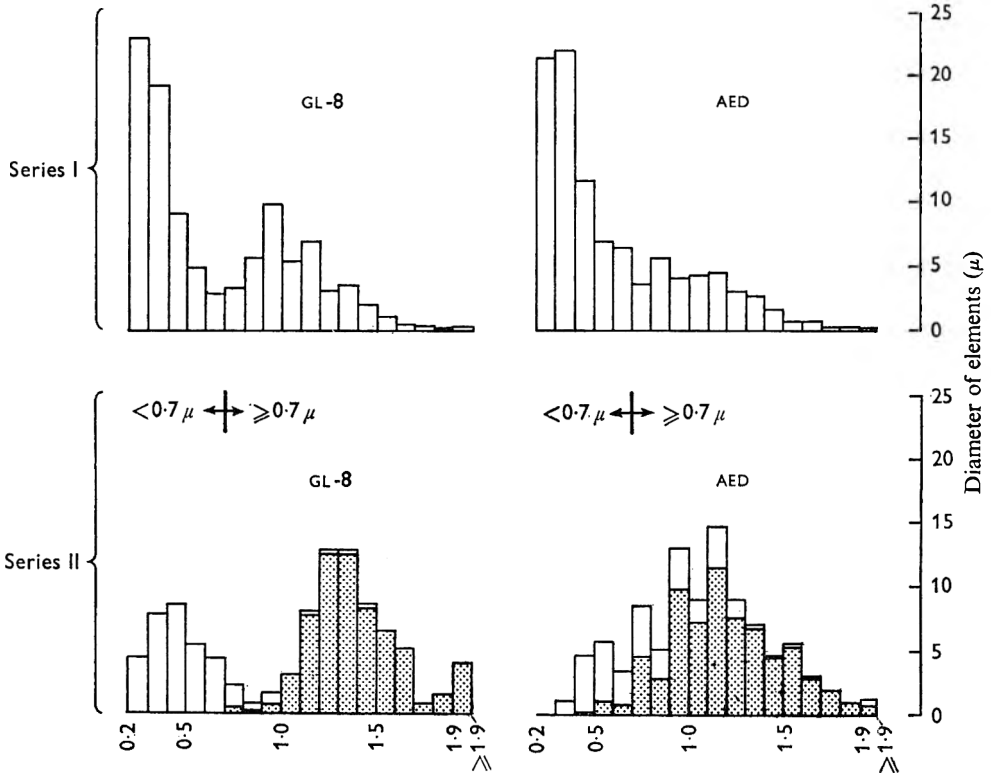

Fig. 1. Histograms of the diameters of L-form elements of the GL-8 and AED strains of group A streptococci. The histograms are based on the figures given in Table 1.

Series I. Frequency distribution of element diameters in 15-hr broth cultures.

Series II. Frequency distribution of the diameters of reproductive ▦ and non-reproductive □ elements in 9-hr cultures.

Spherical and ovoid elements with diameters of about 1 μ and larger are clearly depicted, but the majority of the particles had diameters near the resolving power of the microscope. In the first series of experiments the diameters of the elements were measured on the enlarged photographic prints obtained from these slide cultures. Table 1, series 1 shows the results of measurement of the diameters of 692 and 1445 L-form elements of the GL-8 and AED strains, respectively. In both cases more than half the elements had a diameter of less than 0·5 μ, about one-fourth fall between 0·5–1·0 μ, and the rest showed a diameter of 1·0 μ or larger. The largest elements observed had a diameter of 2·5–3·0 μ.

The frequency distribution of the diameters of the GL-8 L-form elements, as illustrated in Fig. 1, series 1, takes a bimodal form. This bimodality suggests that the

L-form elements can be divided into two classes according to size. These classes were arbitrarily separated at the diameter of 0·7 μ. The bimodality is less clearly shown by the size distribution of the 1445 AED L-form elements.

The average diameter and standard deviation of the two classes of L-form elements were identical for both the GL-8 and AED strain (i.e. 0·4 $\mu \pm 0{\cdot}1$ μ and 1·1 $\mu \pm 0{\cdot}3$ μ). It should be noted that the average diameter and standard deviation of the smaller class are only approximate. The measurements obtained are incomplete because some of the elements of this class probably had diameters below the resolving power of the microscope.

Reproductive capacity of elements of different sizes

The reproductive capacity of the L-form elements, i.e. the capacity to develop into a micro-colony, was assessed in the second series of experiments. Growth was determined by comparing of elements in photographs of the same fields taken immediately after inoculation and at regular intervals during incubation. This is illustrated in Pl. 2, fig. 6, 7, 8. Since L-form cultures of the GL-8 and AED strains yielded a maximal number of viable elements after 9 hr of incubation, in these experiments the slide cultures were inoculated with broth cultures incubated for that period.

Table 1, series 2 shows the results; the total number per class and the number of reproductive elements present are given. The size distribution of the GL-8 L-form elements, as demonstrated by the histogram in Fig. 1 series 2, again indicated the existence of a class of L-form elements with diameters of 0·7 μ or larger, and a class of elements with diameters less than 0·7 μ. The observations for the AED strain were made with a $\times 63$ objective; the resolving power of this system is about 0·5 μ. A small proportion of the class of elements with diameters less than 0·7 μ could therefore be measured. The few measurements obtained below the value 0·5 μ are only approximate.

The elements which developed into a micro-colony had, with a few exceptions in the AED strain, a diameter of 0·7 μ or more. The size distribution of the growing elements (as shown by Table 1, series 2 and by the histograms of Fig. 1, series 2) coincides with the size distribution of the L-form elements belonging to the class of elements with diameters of 0·7 μ or more. The parameters of these size distributions, the mean diameter, and the standard deviation, were the same, i.e. 1·4 $\mu \pm 0{\cdot}3$ μ in the GL-8 strain and 1·2 $\mu \pm 0{\cdot}3$ μ in the AED strain.

Size of reproductive elements in 0·45 μ filtrates

Plate 1, fig. 3, shows a slide culture inoculated with the concentrated filtrate from a 0·45 μ Millipore membrane filter. A large number of particles with diameters near the resolving power of the microscope and a few larger elements are visible. Observation of these slide cultures during incubation showed that only the large elements developed into micro-colonies, as can be seen by comparison of Pl. 1, fig. 3, 4. No growth was observed of any of the very numerous smaller elements. The data on the large elements are limited because, notwithstanding the concentration, the number of these elements was low and they were therefore difficult to find under the microscope. A total of 49 elements which had developed into micro-colonies were observed and photographed. Measurements of the diameter of these elements on the photographs taken immediately after inoculation showed the size of the elements to vary between 0·8 and

1·7 μ. The average diameter and standard deviation of the growing elements, i.e. 1·2 $\mu \pm 0{\cdot}2$ μ, are similar to the values found for the class of reproductive elements.

DISCUSSION

The data in the present paper show a relationship between size as measured microscopically and reproductive capacity of the L-form elements of group A streptococci. A minimal size of 0·7 μ appears to be essential for reproduction, since only elements with a diameter of 0·7 μ or larger developed into micro-colonies. Microscopical studies of the L-form of group A streptococci (Dienes, 1967, 1968) and other bacteria (Roux, 1960; Taubeneck, 1962; Weibull & Lundin, 1962; Weibull, 1963; Fodor & Miltényi, 1964; Dienes, 1968) have also indicated that after transfer to fresh agar medium, growth developed exclusively from elements with a diameter of 0·6–0·7 μ or larger. The pleomorphic character of the L-form is revealed by the variation in size of the viable elements and also by the occurrence of elements with considerably smaller diameters.

The diameters of the streptococcal L-form elements were measured on photographs obtained by phase-contrast microscopy. With phase-contrast microscopy, the resulting modified diffraction gradients usually do not represent the actual boundaries of the objects (Ross, 1957; Ross & Galavazi, 1964). In the present study the 'visible size' was measured according to Chairman (1963) as the distance between the outer visible limits of the diffraction gradients. To assess the error inherent in this procedure, measurements were made on polysterene spheres of known diameter. The results indicate that, at least with particles of this diameter, a reliable estimation of the size of objects can be obtained.

In a previous study (van Boven *et al.* 1968) it has been shown that reproductive elements of the L-form of the same group A streptococcal strains passed through membrane filters with 0·45 μ porosities. The results suggested that passage through this filter might be due to the plasticity of the L-form elements. The demonstration in the present study of L-form elements with diameters between 0·8 and 1·7 μ in the concentrated 0·45 μ filtrates, provides supporting evidence for this assumption. Only these elements were found to develop into micro-colonies; the numerous smaller elements present in the filtrates showed no sign of multiplication during the period of observation. The filterability of the streptococcal L-form might therefore be mainly due to the plasticity of the L-form elements, which would explain the discrepancy between the results of the filtration method and the microscopic measurements with respect to the size of the reproductive L-form elements.

The role of the elements with diameters less than 0·6–0·7 μ is not yet clear. These elements, which range down to 0·05 μ as measured electron microscopically (Thorsson & Weibull, 1958*a*, *b*; Weibull, Mohri & Afzelius, 1965; Ryter & Landman, 1964; Coussons & Cole, 1968), are produced during growth of the bacterial L-form. Dienes (Dienes, 1967, 1968; Dienes & Madoff, 1966, 1968; Dienes & Bullivant, 1967) observed multiplication of these elements when embedded into the agar gel or in the internal structure of membrane filters. In a liquid medium or after transfer to fresh agar medium, multiplication of these elements did not occur; under these circumstances growth developed from larger elements, the so-called 'large bodies'. The latter observation is in accordance with the results of the present study. Confirmation of the former observation cannot be provided by our experiments. The same holds for

the hypothesis put forward by Klieneberger-Nobel (1962) that the small granular L-form elements can develop only when embedded in a matrix of delicate slime. Separation of the granules from the matrix would result in loss of reproductive capacity. The assumption that multiplication of small L-form elements is possible only in the coherence of the L-form colony would explain the failure to demonstrate development of these elements in the present slide-culture experiments. It would also explain the penetration of the streptococcal L-form through membrane filters with low porosities (van Boven *et al.* 1968), but not the filterability of the L-form elements.

Further evidence which seems to exclude the possibility that the 'small' L-form elements play a significant role in the reproduction of the bacterial L-form has been provided by biochemical studies (Weibull & Beckman, 1961; Sensenbrenner, Bader-Hirsch, Terranova & Mandel, 1964) indicating a low DNA content of the small L-phase elements, and by electron-microscopical studies (Ryter & Landman, 1964; Coussons & Cole, 1968) showing the absence of a nuclear region in a considerable number of the small elements.

The bimodality of the size distribution of the group A streptococcal L-form elements, furthermore, suggests the existence of two definite populations. A similar size distribution has been found for the L-form elements of a Proteus L-form (Weibull & Lundin, 1962). The majority of the elements in the size range of 0·6–0·7 μ are apparently capable of growing and increasing in size, which explains the bimodal size distribution.

In conclusion, the results of the previously reported filtration experiments (van Boven *et al.* 1968) and of the present microscopical study indicate a size between 0·6 and 0·7 μ for the minimal reproductive elements of the L-forms of two group A streptococcal strains. Although reproduction of elements of smaller sizes under certain conditions can not be excluded, reproduction of these elements did not occur under the experimental conditions. To elucidate the role of these elements and to further our understanding of the reproductive physiology of the bacterial L-form, detailed analysis of the small elements, especially with respect to the distribution of DNA in the individual elements, will be required.

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EXPLANATION OF PLATES

Photographs of slide cultures of L-forms of Group A Streptococcus prepared according to Taubeneck (1959). The magnification is × 1260.

PLATE 1

Fig. 1. L-form elements of the GL-8 strain of Group A Streptococcus. The slide culture was photographed immediately after inoculation with a 15 hr broth culture.

Fig. 2. L-form elements of the AED strain. Same as Fig. 1.

Fig. 3. Slide culture of GL-8 L-form elements immediately after inoculation. The culture had been filtered through a 0·45 μ filter.

Fig. 4. The same field as in Fig. 3 after 24-hr incubation. The micro-colonies have developed from the two elements with diameters of 0·8 and 1·7 μ.

PLATE 2

Fig. 5. Photograph of control slide culture inoculated with sterile broth.

Fig. 6. Slide culture immediately after inoculation with a 9 hr GL-8 L-form culture.

Fig. 7. The same field as in Fig. 6 after 1 hr incubation.

Fig. 8. The same field as in Fig. 6 and 7. Note that the micro-colonies have developed from the large elements after 3 hr incubation.

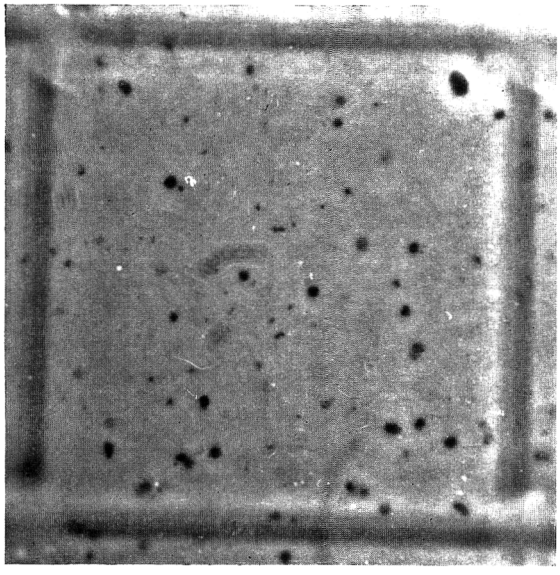

Fig. 1

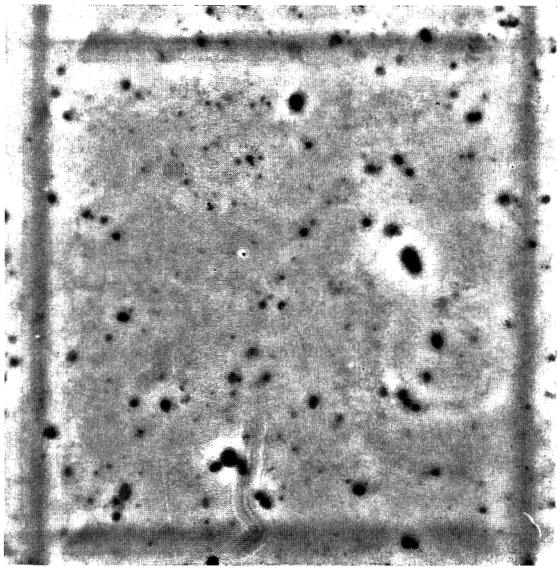

Fig. 2

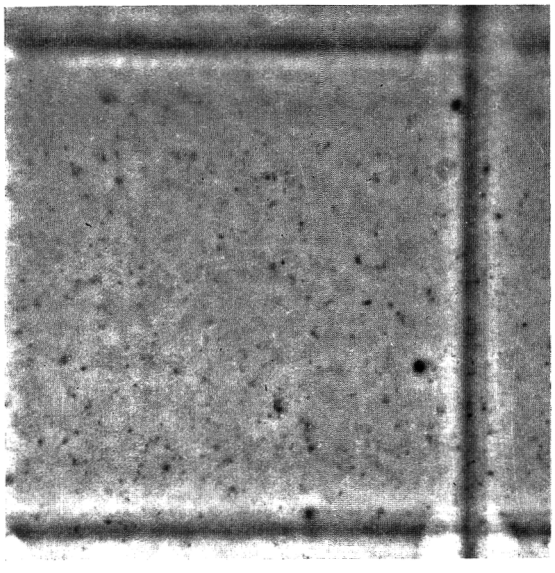

Fig. 3

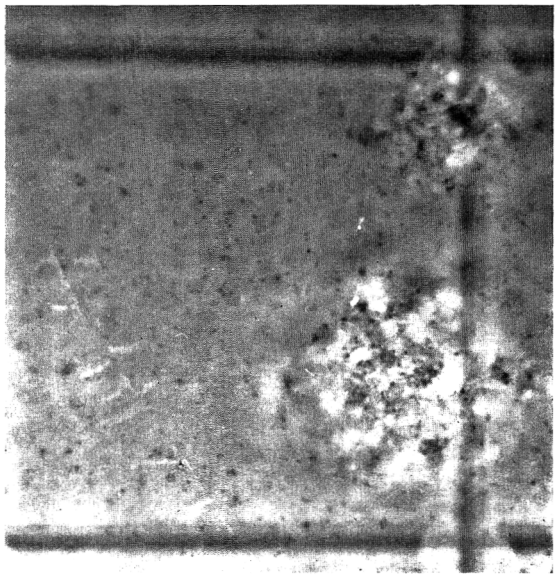

Fig. 4

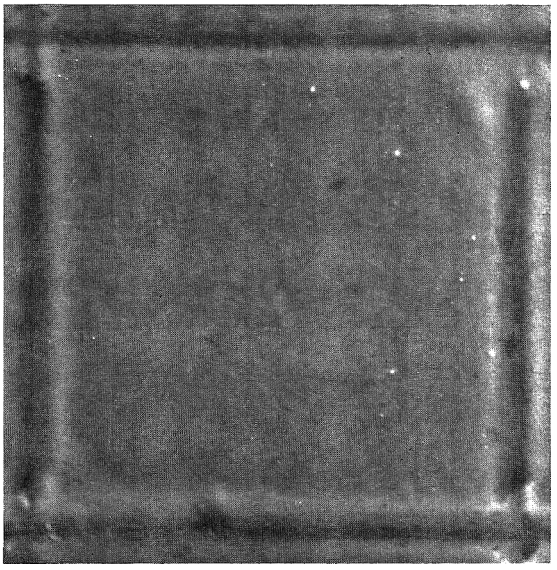

Fig. 5

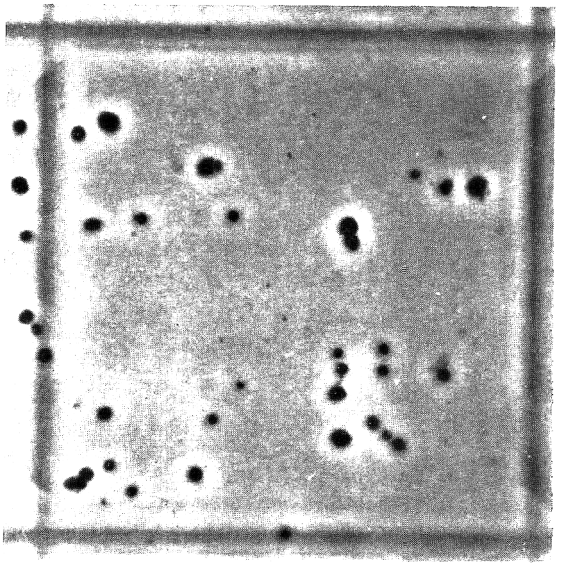

Fig. 6

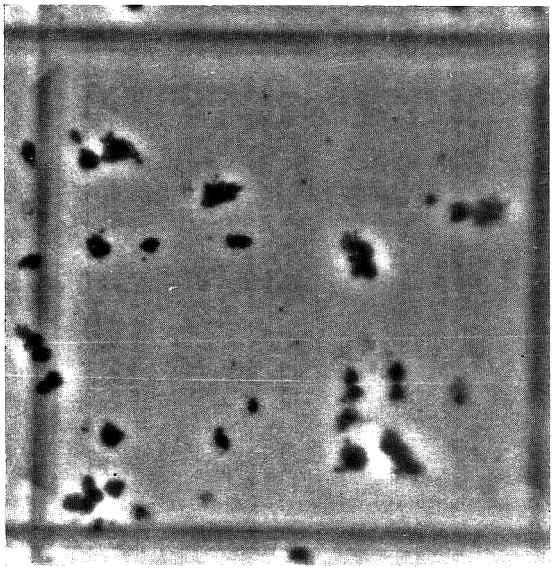

Fig. 7

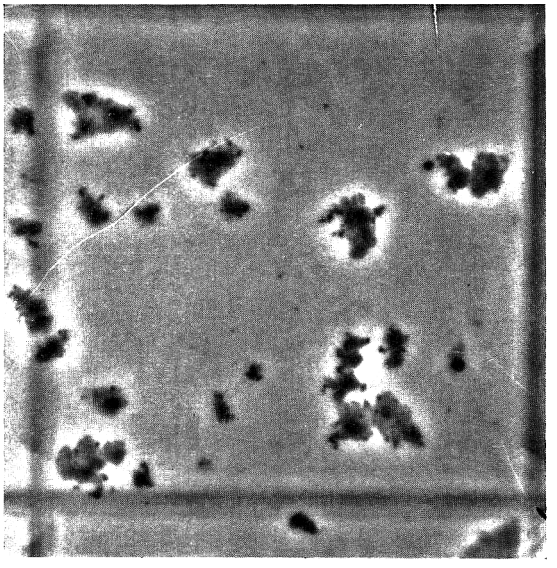

Fig. 8

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Properties of a Basic Amino Acid Permease in *Neurospora crassa*

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SUMMARY

The system for transporting arginine into Neurospora conidia is described and shown to be consistent with the permease concept. The arginine system is shown to be separate from that which transports the aromatic and branched-chain neutral amino acids. Metabolic energy is necessary to achieve a concentration of arginine in the conidia higher than the external medium but not to maintain this concentration differential. A canavanine resistant mutant is shown to transport the basic amino acids at a reduced rate but the mutant transports other amino acids at the normal rate.

INTRODUCTION

Rickenberg, Cohen, Buttin & Monod (1956) described a special type of active transport involving the accumulation of sugars by *Escherichia coli*. They proposed that special molecules, probably protein in nature, bring about the catalytic transport of substances across the cell membrane. They termed these components permeases. The permease concept implied only two essential hypotheses: (1) there is a transient specific complex between the substance to be transported and the permease. (2) The permease is functionally specialized and not involved in intra-cellular metabolism.

The permease concept and the chemical nature of the permease molecule have recently been substantially clarified. Fox & Kennedy (1965), identified a protein component in the cell membrane fraction of *Escherichia coli* as the gene product which functions in the transport of β-galactosides. Kolber & Stein (1966) also identified a protein component in a cell fraction as the product of the β-galactoside permease gene of *E. coli*. Kaback & Stadtman (1966) showed that isolated membranes from *E. coli* could catalyze the transport and concentration of proline. However, membranes from a transport deficient strain did not catalyze the uptake and concentration of proline.

DeBusk & DeBusk (1965) have described a permease system for the transport of phenylalanine in *Neurospora crassa*. They showed that the basic amino acids are poor inhibitors of phenylalanine transport. Stadler (1966) has reported studies on the transport deficiencies of a number of 4-methyltryptophane resistant mutants of Neurospora. These mutants show a large reduction in the transport of the aromatic amino acids and several neutral alaphatic amino acids but minor reduction in the transport of the basic amino acids. Evidence for separate permeases functioning in the transport of arginine and lysine into yeast cells have been reported (Grenson, Mousset, Wiame & Bechet, 1966; Grenson, 1966).

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The purposes of this report are: (1) to describe the arginine transport system in *Neurospora crassa*; (2) to show that there are at least two separate permease systems in Neurospora for the transport of amino acids; (3) to show that arginine and lysine are probably transported by a common permease system; (4) to demonstrate that the energy requiring step for arginine transport operates during the influx process not by altering efflux properties; (5) to show that metabolic energy is required to achieve a concentration of arginine inside the cell greater than the external medium, but not to maintain this concentration differential; (6) to describe some properties of a transport deficient mutant.

METHODS

Strains employed and maintenance of cultures

Neurospora crassa wild-type strains 74A (OR) and SY4f8a were used. Strain 74A was used as the standard in all experiments except those involving canavanine resistance, in which case SY4f8a was the standard wild type. The mutant strain CR-10 was isolated by one of the present authors (W.B.R.). It is an ultraviolet-light-induced mutant derived from wild type SY4f8a. It was initially characterized as a canavanine resistant mutant. Canavanine is an analogue of arginine which will inhibit the growth of strain SY4f8a.

All cultures were grown and maintained on 25 ml. of Vogel minimal medium N (Vogel, 1956) containing 2 % sucrose and 2 % Difco agar. The cultures were either allowed to grow for 5–7 days at 30° or transferred after 24 hr to 25° and allowed to grow for a total of 5–7 days before harvesting. The second procedure was found to improve conidiation. For transport experiments, conidia were harvested, suspended in cold sterile water, and filtered to remove mycelial pieces. Samples were removed and dried to determine the dry weight.

Measurement of amino acid transport

The basic incubation mixture for transport experiments included: (1) Vogel minimal medium N (Vogel, 1956); (2) a ^{14}C uniformly labelled L-amino acid, usually 0·02–0·04 μc/ml. (obtained from Schwarz Bio Research); (3) non-radioactive L-amino acid to adjust the concentration of the amino acid to the desired specific activity, which was usually either 0·5 or 0·25. The experiments were initiated by adding the conidial suspension to the incubation mixture which was previously adjusted to 30°. The conidia were kept in suspension by shaking in a constant temperature water bath.

Samples (5 ml.) were removed from the incubation mixture at programmed times and deposited on a membrane filter (millipore HA 0·45 μ, 25 mm). The samples were washed with 5 volumes of water and glued to a planchet for later counting in a Baird Atomic thin window gas flow proportional counter. The results in the text, unless otherwise indicated, express the total amount of radioactive amino acid taken up by the conidia.

Measurement of intra-cellular pools

When measuring the free amino acid pool two 5 ml. samples were removed from the basic mixture. The first sample was immediately filtered and washed as previously described. The second sample was added to 10 ml. of 10 % cold trichloroacetic acid (TCA) and after 30 min. the cells were filtered and washed in the usual fashion. From differences in the radioactivity between these samples the size of the free pool was determined.

Measurement of concentration gradient

The conidial volume of a 5 ml. sample (taken from the usual incubation medium for a transport experiment) was determined from the packed cell volume of the original concentrated suspension of conidia. The ratio of the free intracellular arginine to that remaining in the incubation mixture after the conidia had been removed gave an approximation of the gradient established at a particular time.

The magnitude of the concentration gradient was also determined in another way. Hot water extracts of conidia were analysed on a Beckman amino acid analyser. The conidia (5–6 mg.) were suspended in 5 ml. H_2O after a 3 hr incubation in the basic incubation mixture containing L-arginine-^{12}C. The control conidia were incubated without arginine. The conidia were boiled for 25 min. and then centrifuged. The supernatant was decanted, chilled, recentrifuged and adjusted to pH 2·1 with 0·1 N-HCl to a final volume of 5 ml. Part of this sample was then passed through the amino acid analyser. The difference in arginine concentration between the experimental value and that obtained with control conidia was used to determine the concentration of free arginine which was accumulated in a unit volume of packed conidia.

Chromatograpny of hot water extracts

Following an incubation in L-arginine-^{14}C, amino acids were extracted from conidia with hot water. These extracts were chromatographed employing one dimensional paper chromotography with water-saturated phenol used as a solvent. The chromatogram strips were developed with ninhydrin and the radioactivity was determined by counting the strips in a windowless Nuclear-Chicago Actigraph.

RESULTS

Transport of arginine into conidia of *Neurospora crassa* is temperature dependent. The temperature optimum for the arginine transport system occurs between 30° and 40° (Fig. 1). The rates of transport at 40° and 50° were not constant but declined as the incubation proceeded, indicating an inactivation of the transport system at these temperatures. At zero degrees there was no accumulation of arginine in the cells above the concentration in the medium.

The transport of arginine in Neurospora is also pH dependent. The optimal pH is about 5·6 (Fig. 2). The results illustrated in Fig. 2 were obtained from experiments in which potassium phosphate solutions (0·1 M) were used as the incubation media. Using other buffers (citrate, tris, sodium phosphate) over small pH ranges it was confirmed that the optimal pH for arginine transport is between 5 and 6. Since Vogel minimal medium is buffered in this range it was used in all subsequent experiments as the incubation medium.

Effect of the external arginine concentration

At relatively low arginine concentrations the rate of transport was shown to depend on the concentration in the medium. However, the rate was not increased by raising the arginine concentration above 0·6 μmoles/30 ml. (Fig. 3). In this experiment each ml. of incubation medium contained 0·1 mg. (dry wt) of conidia.

Initial velocities of arginine transport were obtained in an experiment similar to that

illustrated in Fig. 3, but in this second experiment lower concentrations were used. The data obtained from samples incubated for 5 and 10 min. were considered to represent initial velocities, since there was no change in the transport rate during this time. The data were plotted according to the methods of Lineweaver & Burk (1934) (Fig. 4). From these experiments a typical Km of 2×10^{-6} M was obtained. This value corresponds to a concentration of 0·002 μmoles/ml. Independent determinations of a Km value have varied between $1{\cdot}74 \times 10^{-6}$ M and $2{\cdot}1 \times 10^{-6}$ M. In each case points for the highest concentrations do not lie on the line as drawn in Fig. 4. We have no explanation for this but it is typical of plots showing high substrate inhibition.

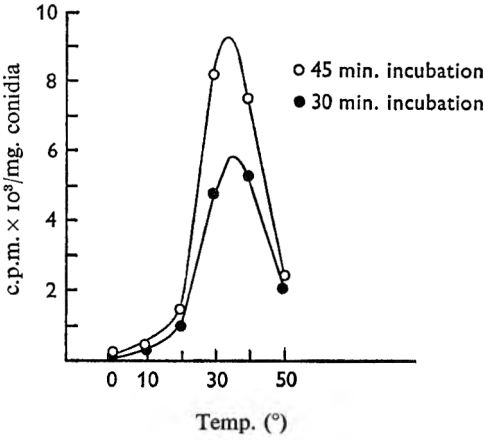

Fig. 1

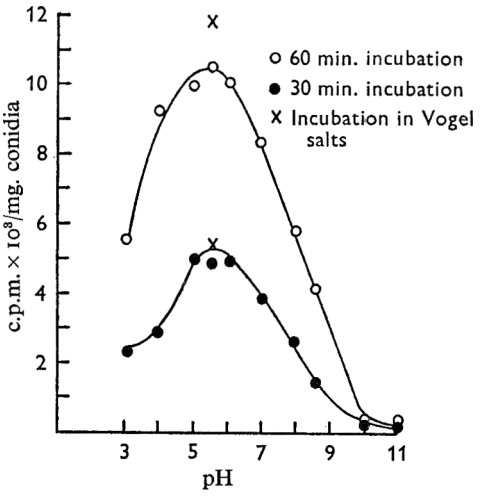

Fig. 2

Fig. 1. Arginine transport as a function of temperature. The basic incubation mixture contained 0·114 mM-L-arginine-^{14}C with a specific activity of 0·35 μC/μ mole. The conidial concentration was 0·06 mg. (dry wt)/ml.

Fig. 2. Arginine transport as a function of pH. Potassium phosphate (0·1 M) solutions were used as the incubation mixtures containing 0·08 mM-L-arginine-^{14}C with a specific activity of 0·5 μC/μ mole. The conidial concentration was 0·08 mg. (dry wt)/ml. in a total initial volume of 25 ml. The incubations in Vogel salts were for 30 and 60 min.

Measurement of transport gradient

Arginine can be transported against a concentration gradient. Under the conditions of limited incorporation of amino acids into protein (incubation without glucose or with actidione added to inhibit protein synthesis) the arginine pool was near its maximum size after 3 hr of incubation. The ratio of arginine in the pool of a unit volume of packed conidia to the concentration remaining in an equal volume of the incubation medium was calculated to be 314. Hot water extracts of the conidia were chromatographed to establish the percentage of radioactivity which was still associated with arginine. These results showed that less than 5 % of the arginine was converted to other forms. There was a small amount of radioactivity in the ornithine spot on the chromatogram and in another spot which was not identified but was probably urea. The arginine concentration value in the hot water extracts was also obtained by passing a sample (non-radioactive) through an amino acid analyser. In this case the concentra-

tion of arginine in conidia incubated in medium containing arginine was shown to be 15 μmoles/ml. of packed conidia above the control level. The ratio of arginine concentration in the cells to that in the medium was 444. The amount of radioactivity which was not extractable with hot water or cold trichloroactic acid (TCA) was used to indicate what percentage of the total arginine taken up was incorporated into protein. Under standard conditions (no glucose added) the amount incorporated into TCA insoluble material was less than 15 %.

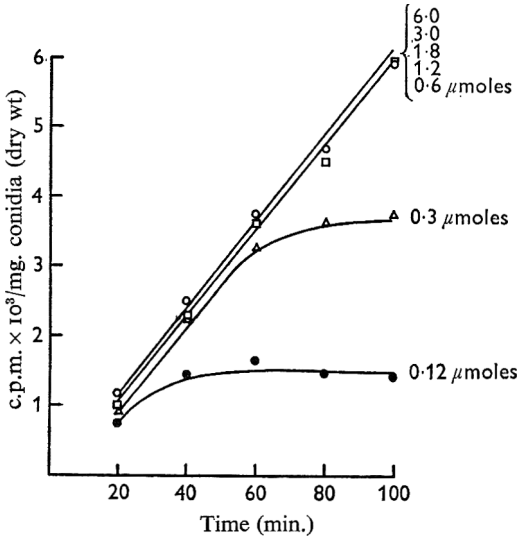

Fig. 3

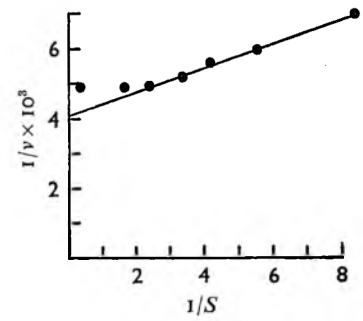

Fig. 4

Fig. 3. Arginine transport as a function of external arginine concentration. The basic incubation mixture (30 ml.) contained L-arginine with a specific activity of 0·17. The concentrations indicated are expressed as μmolcs/30 ml. The conidial concentration in each case was 0·1 mg (dry wt)/ml.

Fig. 4. Lineweaver–Burk plot of L-arginine transport. The initial velocities are expressed as μmoles of L-arginine transported per mg. dry wt/minute. The substrate concentrations are expressed as μmoles per 40 ml.

The energy requirement in the transport process

The transport of arginine was inhibited by 2,4-dinitrophenol (0·42 mM). This compound immediately inhibited arginine transport if added after 40 min. or added at the beginning of the incubation (Fig. 5). It can also be seen (Fig. 5) that there is little or no efflux of the arginine from the cells after the energy generating system is inhibited. Nearly identical results were obtained when sodium azide was used as the inhibitor. Since the mechanism of inhibition of oxidative phosphorylation by 2, 4-DNP and NaN_3 is not clearly understood, another inhibitor was used to determine what effect the loss of ATP formation has on arginine transport. Antimycin A inhibits the reoxidation of coenzyme Q of the electron transport system (Green, Hatefi & Fechner, 1959). This antifungal agent caused a similar inhibition of arginine transport (Fig. 6). Attention is again directed to the lack of efflux of previously accumulated arginine when an ATP generating system was inhibited. Under nearly identical conditions DeBusk & DeBusk

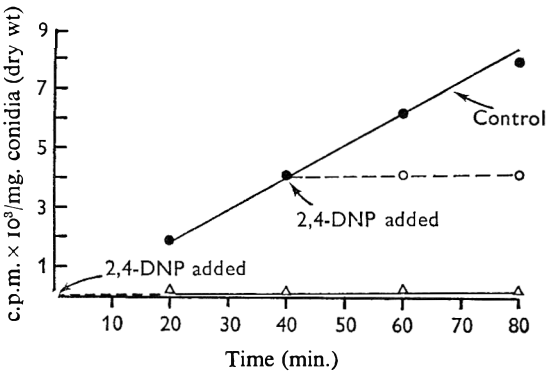

Fig. 5

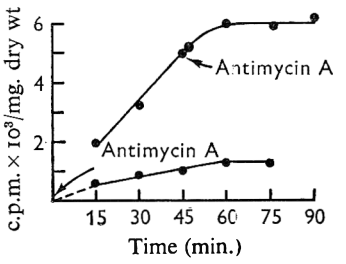

Fig. 6

Fig. 5. The effect of 2,4-dinitrophenol (2,4-DNP) on arginine transport. The basic incubation mixture (30 ml). contained 0·03 mM-L-arginine-^{14}C with a specific activity of 0·25 μC/μmole. The conidial concentration was 0·06 mg. (dry wt)/ml. The concentration of 2,4-DNP was 0·42 mM.

Fig. 6. The effect of antimycin A (0·01 mg./ml). on arginine transport. The basic incubation mixture (50 ml.) contained 0·04 mM-L-arginine with a specific activity of 0·5 μC/μmole. The conidial concentration was 0·04 mg. (dry wt)/ml.

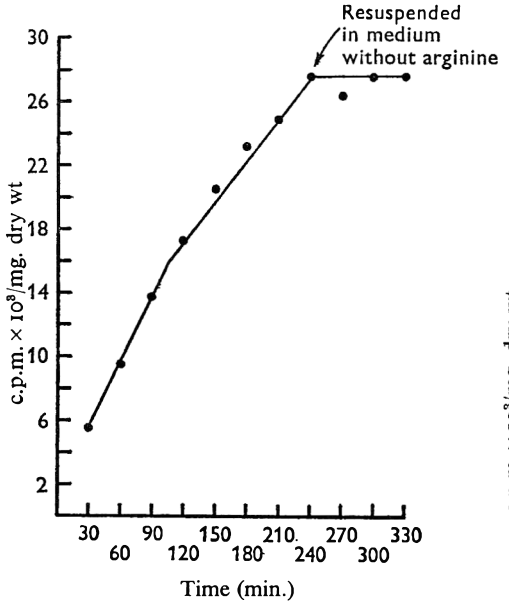

Fig. 7

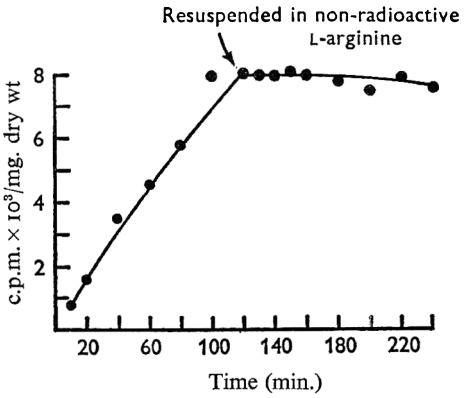

Fig. 8

Fig. 7. Lack of efflux of arginine after resuspending in medium without arginine. The basic incubation mixture (100 ml.) contained 0·08 mM-L-arginine-^{14}C with a specific activity of 0·5 μC/μmole. The conidial concentration was 0·06 mg. (dry wt)/ml. The actidione concentration was 0·2 mg./ml.

Fig. 8. Lack of exchange of pool arginine with arginine in the external medium. The basic incubation medium (100 ml.) contained 0·16 mM-L-arginine-^{14}C with a specific activity of 0·25 μC/μmole. The conidial concentration was 0·05 mg. (dry wt)/ml. The cells were resuspended in 0·16 mM-L-arginine-^{12}C.

(1965) found that phenylalanine was not maintained in the cells when NaN_3 and 2-4-DNP were added.

The lack of efflux of accumulated arginine was further examined. Conidia incubated for 4 hr in the basic medium containing radioactive arginine were resuspended in medium without arginine. No efflux was observed (Fig. 7). This experiment was carried out with actidione added to stop protein synthesis. Similar results were obtained when cells were incubated for only 3 hr before resuspending them.

Efflux could not be demonstrated in the absence of an external source of arginine even though a large arginine pool was maintained by the cells. The question remained whether the arginine in this pool would exchange with the arginine in the external medium. To answer this question, conidia were incubated in radioactive arginine for 2 hr. They were removed from the medium containing radioactive arginine, washed with distilled water and resuspended in medium with unlabelled arginine. As seen in Fig. 8, no efflux or exchange of the arginine in the cells with that in the external medium was obtained.

Determination of stereospecificity and inhibition by L*-amino acids*

The transport of L-arginine was not affected by the addition of D-arginine. Table 1 shows that the addition of non-radioactive L-arginine reduced the transport of the labelled L isomer, whereas the D-isomer did not.

Table 1. *Effect of* D*- and* L*-isomers of arginine on* L*-arginine transport*

Additions*	Control	1 μmole L-arginine-^{12}C	1 μmole D-arginine-^{12}C	5 μmoles D-arginine-^{12}C
Time of incubation in minutes	L-Arginine uptake expressed as counts per minute/mg. dry wt			
15	2,800	1,518	3,180	2,798
30	7,200	4,000	7,600	6,930
45	11,600	6,000	11,500	10,700
60	15,700	8,100	15,100	14,400

* Each incubation flask contained 0·04 mM-L-arginine-^{14}C with a specific activity of 1 μC/μmole in 25 ml. of the basic medium.

Many of the naturally occurring amino acids inhibit the transport of arginine. Table 2 shows the results of amino acid inhibition experiments. The inhibiting amino acids were present at concentrations of 20, 50 and 100 μmoles per 25 ml. in combination with one μmole of L-arginine. These results show that the basic amino acids lysine and ornithine were the best inhibitors; glutamic acid, a dicarboxylic amino acid, was a poor inhibitor; and the neutral amino acids were intermediate. Proline, an imino acid, was not inhibitory to arginine transport.

In order to determine if the uptake inhibition by non-basic amino acids was occurring at the entry site or through a competition for a common pool, the following experiment was done. Conidia were incubated for varying times in the basic medium containing phenylalanine. The cells were removed from the phenylalanine medium, washed and suspended in medium with labelled arginine. The rate of arginine transport was examined and compared in these cells containing varying phenylalanine pool concentrations. The phenylalanine pool concentrations were previously measured and shown to increase linearly over the first 60 min. of incubation (DeBusk & DeBusk,

1965). Table 3 shows that phenylalanine previously accumulated by the cells did not affect the transport of arginine. This result indicated that the inhibition of arginine by phenylalanine appeared to be at the transport level rather than at the pool level. The term 'pool' is defined as, that concentration of amino acid which can be extracted from the cell under conditions which cause no degradation of macromolecules.

Table 2. *Effects of other amino acids on the transport of* L-*arginine in wild type strain 74A. Arginine uptake expressed as percentage of control*

Inhibiting amino acid	10:1*	20:1	50:1	100:1
Lysine	28·8	15·2	—	5·2
Ornithine	—	29·6	18·3	12·0
Histidine	—	40·0	27·5	28·0
Phenylalanine	43·5	42·6	—	33·6
Tryptophan	—	32·0	32·0	27·5
Citrulline	—	55·4	41·6	36·0
Alanine	55·8	49·0	—	41·0
Isoleucine	—	54·0	44·5	37·2
Leucine	—	46·8	41·0	42·2
Methionine	—	44·5	40·0	39·2
Serine	—	68·0	55·0	47·0
Glutamic acid	—	96·0	78·0	54·0
Glycine	—	75·5	60·3	53·0
Threonine	—	73·0	65·0	50·0
Proline	—	101·0	98·2	101·0

* Ratio of inhibiting amino acid concentration to arginine concentration. The L-arginine-^{14}C concentration in each case was 0·04 mM with a specific activity of 0·5 μC/μmole.

Table 3. *Effect of phenylalanine pool size on arginine transport*

Time of incubation (min.)*	Minutes of previous incubation in the basic medium containing 0·08 mM-DL-phenylalanine				
	0	10	20	30	40
	L-Arginine uptake expressed as counts per minute/mg dry wt				
10	1,680	1,740	1,960	1,925	1,970
20	3,360	3,225	3,560	3,410	3,360
40	6,250	6,100	6,790	6,170	6,050
60	8,790	8,650	8,650	9,000	8,430
80	11,100	10,200	11,780	10,750	10,810

* Each incubation flask contained 0·08 mM-M-L-arginine-C^{14} with a specific activity of 0·5 μC/μmole in 50 ml. of the basic medium.

Simultaneous transport experiments in wild type 74A

The specificity of the system transporting arginine needed further investigation. If separate systems existed for the transport of two different amino acids, it was reasoned that the accumulation of radioactivity by conidia incubated with two radioactive amino acids would be greater than the rate for either amino acid independently. However, the rate would be intermediate between the separate rates if the two amino acids were transported by a common system. The concentration of each amino acid in the medium had to be sufficiently high so that the separate rate of transport of the amino acid was independent of the external concentration.

When lysine-^{14}C and arginine-^{14}C were simultaneously transported from the same incubation mixture, the resulting rate of ^{14}C accumulation was an average of the separate rates for arginine and lysine (Fig. 9). This indicates that arginine and lysine are transported by a common permease system. The results were quite different when the rates of transport of arginine-^{14}C in combination with either tyrosine-^{14}C, leucine-^{14}C, or phenylalanine-^{14}C were examined. In each of these cases the combined initial

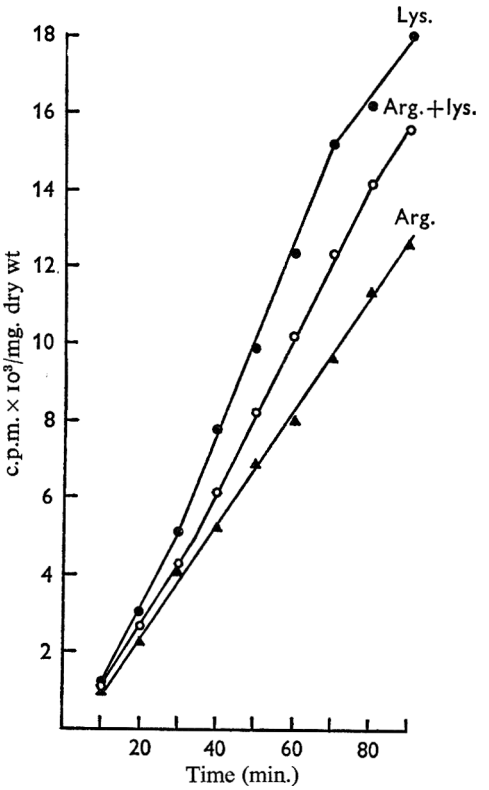

Fig. 9

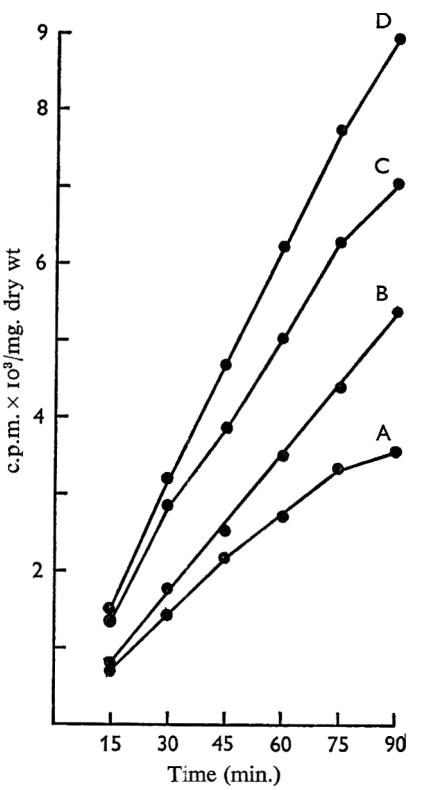

Fig. 10

Fig. 9. Comparison of the simultaneous and separate transport rates of L-arginine and L-lysine in a wild type strain 74A. The basic incubation mixture (50 ml.) contained respectively:

(1) simultaneous transport; 0·08 mM-L-arginine-^{14}C with a specific activity of 0·5 μC/μmole; 0·08 mM-L-lysine-^{14}C with a specific activity of 0·5 μC/μmole.

(2) Arginine transport alone; 0·08 mM-L-arginine-^{14}C with a specific activity of 0·5 μC/μmole.

(3) lysine transport alone; 0·08 mM-L-lysine-^{14}C with a specific activity of 0·5 μC/μmole.

(4) the concentration of conidia in each case was 0·07 mg. (dry wt)/ml.

Fig. 10. Comparison of simultaneous and separate transport rates of L-arginine and L-tyrosine. The basic incubation mixtures (50 ml.) contained respectively:

Curve A. L-tyrosine-^{14}C (0·08 mM) with a specific activity of 0·25 μC/μmole.

Curve B. L-arginine-^{14}C (0·08 mM) with a specific activity of 0·25 μC/μmole.

Curve C. L-tyrosine-^{14}C (0·8 mM) with a specific activity of 0·25 μC/μmole plus L-arginine-^{14}C (0·08 mM) with a specific activity of 0·25 μC/μmole.

Curve D is the calculated sum of Curve A + Curve B. The conidial concentration in each case was 0·07 mg. (dry wt)/ml.

rate was clearly greater than either of the two separate rates and closely approached a calculated sum of the separate rates. A typical result is illustrated in Fig. 10.

The transport of phenylalanine-^{14}C in combination with leucine-^{14}C or tyrosine-^{14}C resulted in each case in a ^{14}C accumulation rate intermediate between the separate rates. This result suggests that phenylalanine, tyrosine and leucine are transported by a common permease. These results are consistent with the inhibition results for phenylalanine transport reported by DeBusk & DeBusk (1965) and the growth inhibition experiments of Brockman, DeBusk & Wagner (1959).

Properties of a canavanine resistant mutant

If, as indicated by the above results, a specific permease functions in the transport of the basic amino acids, a mutant should be obtainable which has reduced transport capacity for only the basic amino acids. In order to find such a mutant we isolated strains which would grow on canavinine (an analogue of arginine). One of the mutants designated CR-10 fits this description (Table 4). Arginine, lysine and histidine were transported at reduced rates whereas other selected amino acids and a glucose analogue, 3,0-methylglucose were transported at the normal rate.

Table 4. *Comparison of transport between wild type strain SY4fga and mutant strain CR 10**

Time (min.)	Arginine		Lysine		Histidine		Phenylalanine		Leucine	
	Wt	CR 10	Wt	CR 10	Wt	CR 10	Wt	CR 10	Wt	CR 10
15	970	565	2421	1504	1389	901	1759	2143	1487	1804
30	1710	1017	4395	2713	2303	1659	2662	3261	2680	3270
45	2453	1500	6083	4213	3180	2360	3197	3840	3640	4048
60	3200	2022	8004	5152	3930	2810	3618	4130	3947	4970
75	3910	2467	9754	6235	4643	3462	3360	3973	4364	4930
	Alanine		Serine		Glycine		Aspartic acid		3-methyl glucose	
	Wt	CR 10	Wt	CR 10	Wt	CR 10	Wt	CR 10	Wt	CR 10
15	1266	1630	1209	1397	1727	1835	149	151	496	430
30	2330	2810	2277	2526	3125	3626	273	250	623	570
45	3314	3594	3341	3793	4448	4905	360	328	1053	904
60	4189	4909	4215	4504	5820	6672	466	474	1456	1307
75	5138	6194	5406	5582	7262	8332	615	608	1890	1680

* All experiments conducted with 5 mg./ml. of actidione added. The specific activity of each amino acid was the same (1 μC/4 μmoles) except lysine (2 μC/4 μmoles). O-methyl-glucose specific activity was 1 μC/10 μmoles. All results are expressed as counts/minute/mg. dry wt conidia.

DISCUSSION

Evidence had been presented here to support the conclusion that arginine is actively transported across the cell membrane of *Neurospora crassa* conidia by a constitutive stereospecific system. Establishment of a 400-fold difference in the concentration inside the conidia over the external medium was demonstrated. The transport process which established this gradient was shown to require metabolic energy and is dependent on the pH of the medium and the temperature. This transport system also showed saturation kinetics typical of enzymic reactions.

Koch (1964) and Winkler & Wilson (1966), studying galactoside transport in *Escherichia coli*, proposed that the energy step in active transport brings about a decrease in the efflux rate of the galactoside resulting in a net increase in influx. In Neurospora previously accumulated arginine does not efflux in the absence of external arginine or exchange with arginine in the medium, and no efflux is observed when energy poisons are employed. Therefore a different explanation involving energy utilization must be proposed for arginine accumulation in Neurospora.

The experiments described here suggest that the energy step in arginine transport is directly involved in influx, not in reducing an efflux system. This implies that energy is expended to achieve a high concentration gradient of arginine inside the cell but not to maintain it.

The results of the experiments involving the simultaneous transport of two labelled amino acids argue for the existence of at least two separate permeases. Each of these permeases is thought to be shared by separate groups of amino acids. One group composed of the basic amino acids and the other the branched and aromatic neutral amino acids.

The simultaneous rates were not in any case completely equal to the sum of the separate rates. This observation along with the observed interference by the non-basic amino acids on arginine transport would indicate that some overlap in function of the separate permeases exists or there is an additional less specific transport system. Another likely explanation is that the cross inhibitions which are observed occur at a secondary step subsequent to the interaction of the permeases with the amino acids.

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The Effect of 2-deoxy-D-Glucose on the Growth and Respiration of *Coprinus lagopus*

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SUMMARY

The unnatural glucose analogue 2-deoxy-D-glucose was found to be fungistatic rather than fungicidal for a laboratory wild-type strain of the Basidiomycete *Coprinus lagopus*; the analogue was not used as a carbon+energy source. Extension-growth rate of the organism was profoundly affected by the presence of 2-deoxyglucose; but although this growth could be prevented completely the inhibition was readily annulled, either by the addition of an excess of the normal substrate or by transfer to normal media. The extent of the inhibition observed depended not only on the ratio of normal hexose to analogue, but also on the identity of the normal hexose in the medium. *Coprinus lagopus* was at least 100 times more sensitive to the analogue when fructose was present than with glucose. This extreme sensitivity differential was also observed in the effect of 2-deoxyglucose on oxygen uptake by spore suspensions: inhibition of O_2 uptake occurred, and was about 100 times greater with fructose than with glucose.

INTRODUCTION

Woodward, Cramer & Hudson (1953) reported that the unnatural glucose analogue 2-deoxy-D-glucose (deGlc) caused inhibition of growth of Saccharomyces. This was confirmed by Heredia, de la Fuente & Sols (1964) who showed that growth and glycolysis were inhibited in *Saccharomyces cerevisiae* and *S. fragilis*. These workers showed that growth on glucose+deGlc (2 %+0·1 %) was only about 30 % of that on normal hexoses, while virtually no growth was made on fructose+deGlc at the same concentrations. Incubation of organisms with fructose+deGlc lead to the death of over 95 % of active organisms in about 24 hr.

The growth of *Neurospora crassa* was decreased on media containing equimolar concentrations of deGlc and glucose, though with limiting glucose the addition of analogue increased the final growth yield (Sols, Heredia & Ruiz-Amil, 1960). *Neurospora crassa* can utilize deGlc as sole carbon+energy source although the growth is weak. On the other hand *Aspergillus oryzae* utilizes deGlc fairly readily, the growth rate being about one fifth of that on normal hexose (Sols *et al.* 1960). Barban & Schulze (1961) working with human cell cultures, showed that there was essentially no growth at equimolar concentrations of deGlc+glucose and that after about 3 days of exposure to this medium the cytopathogenic effects were irreversible, cells grown on fructose were 5 to 10 times more sensitive to inhibition than those grown on glucose. The respiration of yeasts (Heredia *et al.* 1964) and human cells (Barban & Schulze, 1961) was markedly inhibited by deGlc. In the case of yeasts the inhibition of respiration was not as great as the inhibition of growth and was not a direct cause of the latter.

Enzymic studies have shown that deGlc is readily phosphorylated by hexokinase (ATP: D-hexose 6-phosphotransferase, E.C. no. 2.7.1.1.) (Heredia *et al.* 1964; Barban & Schulze, 1961; Sols *et al.* 1960) without inhibiting glucose phosphorylation (Barban & Schulze, 1961). However, the phosphorylation of fructose was actively inhibited by deGlc in HeLa cell extracts. Phosphohexoseisomerase (D-glucose-6-phosphate ketol-isomerase, E.C. no. 5.3.1.9.) and glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate: NADP oxidoreductase, E.C. no. 1.1.1. 49.) from HeLa cells were both inhibited by deGlc-6-phosphate (Barban & Schulze, 1961). The available evidence indicates that the inhibitory effects of deGlc are conditional on its phosphorylation. Inhibition of the entry of normal sugars into the cell by accumulated deGlc phosphates and possibly inhibition of early enzymes of glycolysis and of the pentose phosphate cycle are probably sufficient to account for the effects seen on respiration. The lethal effect observed with yeasts (Heredia *et al.* 1964; Megnet, 1965) is probably accounted for by a trapping of uridine nucleotides by phosphorylated derivatives of deGlc, with a consequent interference with the synthesis of structural polysaccharides (Heredia *et al.* 1964). A deGlc-resistant mutant of yeast (Heredia & Sols, 1964) and a resistant sub-line of HeLa cells (Barban, 1962) have been described. In both cases growth (40 % to 50 % of normal) was obtained with equimolar concentrations of normal hexose + deGlc—conditions under which the parental strains showed no growth. The evidence indicated that resistance in each case was due to an abnormally high phosphatase activity which decreased the intracellular deGlc phosphate concentration. The general effects of deGlc in a wide range of systems were reviewed by Webb (1966).

The present paper describes work on the effects of deGlc on the Basidiomycete *Coprinus lagopus*. The particular interest of this organims lies in the observation that it is naturally highly resistant to the analogue in the presence of glucose, but very sensitive in the presence of fructose.

METHODS

The organism used was a strain of *Coprinus lagopus*, isolation number BC9/66; it is a haploid prototrophic laboratory wild type. It was maintained by monthly sub-cultures on complete medium.

Media. The basal medium (minimal medium) had the following composition (Analar grade reagents): asparagine, 13 mM; ammonium tartrate, 3·3 mM; Na_2HPO_4, 10 mM; KH_2PO_4, 10 mM; Na_2SO_4, 2 mM; thiamine hydrochloride, approximately 1×10^{-4} % (w/v). Hexoses were added to this medium in the concentrations indicated in the various experimental descriptions; normal hexoses were obtained from British Drug Houses Ltd., and 2-deoxy-D-glucose from the Sigma Chemical Company (London). A fully supplemented (complete) medium was prepared by adding glucose (to 100 mM), Bacto Casamino acids (0·07 %, w/v), Bacto yeast extract (0·07 %, w/v), Bacto malt extract (1·0 %, w/v), and hydrolysed nucleic acids (approximately 0·04 %, w/v) to the above. Media were solidified with Bacto agar (1·5 %, w/v).

Preparation of oidial suspensions. In general monokaryotic strains of Coprinus produce asexual spores (oidia) continuously during the growth of the colony. These are formed by aerial hyphae and can be found in abundant numbers in fluid droplets in the aerial mycelium. Oidia were harvested from slope cultures of *Coprinus lagopus* BC9/66 on complete medium made up in 6 in. × 1 in. Pyrex glass boiling tubes. Ten ml. of distilled water were added to a tube after at least 5 days of incubation at 37°, and

the oidia scraped into suspension with an inoculation needle or loop. After filtration through cotton-wool to remove mycelial debris the concentration of oidial suspensions was estimated with the aid of a haemacytometer slide. Viability of suspensions was determined by plating samples on complete medium agar and assessing what proportion of the visible oidia was able to grow into colonies. Where, in the sequel, results are given in terms of spore numbers these refer to the number of viable oidia, not to the total number of visible oidia.

Agitated suspensions. In the majority of experiments oidia were treated in suspension. The suspensions (10 ml.) were contained in 1 oz. McCartney bottles and continuously agitated by mounting on an angled turn-table within an incubator at 37°. At intervals 1 ml. samples were removed, diluted and spread on to plates of complete medium. Plates were incubated at 37° and colonies counted after about 48 hr.

Growth rates. The rate of extension growth was determined by measuring colony diameters at successive stages during incubation. Growth was initiated with 2 mm. diam. disks cut with a punch from established (about 3 days old) complete medium cultures. Conditions of growth were standardized as far as possible; media were prepared and sterilized in one batch, 20 ml. quantities were used in plastic Petri dishes, and inoculated plates were incubated at 37° together in a single incubator. Three replicates of each treatment were measured over two diameters at right angles.

Measurements of oxygen uptake. The rate of oxygen uptake by oidia in suspension was measured by using a conventional Warburg manometric apparatus at 37°. Warburg vessels contained in their main compartment, minimal medium constituents, oidia, and the required hexoses all in a total volume of 3·0 ml. All additions were adjusted in amount to give the required concentrations in this final volume. Oidial suspensions were adjusted to give a final concentration of 3×10^8 viable oidia/ml.

RESULTS AND DISCUSSION

Viability of oidia

The fungicidal action of 2-deoxy-D-glucose (deGlc) was first investigated by an agar overlay technique. Oidial viability was compared by incubating spores at 37° on minimal medium containing 100 mM-glucose and on minimal medium containing 100 mM-glucose + 5 mM-deGlc. At 8 hr intervals the plates were overlaid with complete medium, re-incubated until colonies appeared and these then counted. Survival was uniformly good (75–100 %) there being no significant difference between survival on the two types of medium even in those samples which had been exposed to deGlc for 48 hr. This technique was abandoned in favour of one using agitated oidial suspensions when it became clear that oidia would germinate and grow vigorously on minimal medium + 100 mM-glucose + 5 mM-deGlc. Medium overlaid on plates which had been incubated for more than about 48 hr spread secondary oidia derived from established colonies, thus confusing the results.

The survival of oidia following treatment in suspensions containing various concentrations of glucose and deGlc is shown in Fig. 1. Quantitative results were not reliable for most treatments of duration longer than about 25 hr, because of the appearance of colonies within the suspensions. Such colonies both trapped oidia and prevented accurate pipetting. Well-developed colonies were visible at the 25 hr sample time in the controls and in those experiments containing 5 mM-deGlc + 10 mM-glucose;

at 32·5 hr in 25 mM-deGlc + 10 mM-glucose; and small colonies were also seen in 50 mM-deGlc + 10 mM-glucose at 32·5 hr. Very small colonies were also visible in the other experimental bottles at the end of the experiments. Despite this difficulty, however, the data clearly show that there was no very significant effect (if any) on oidial survival even at a 50:1 molar ratio of deGlc:glucose. Furthermore, the appearance of colonies in the suspensions indicates that the coprinus was highly resistant to deGlc under these conditions.

Tests for oidial survival in liquid media containing fructose and deGlc were delayed until the extension-growth experiments were completed (see below). Survival in media containing 5 mM fructose + 0·5 mM-deGlc was then examined, since growth was known

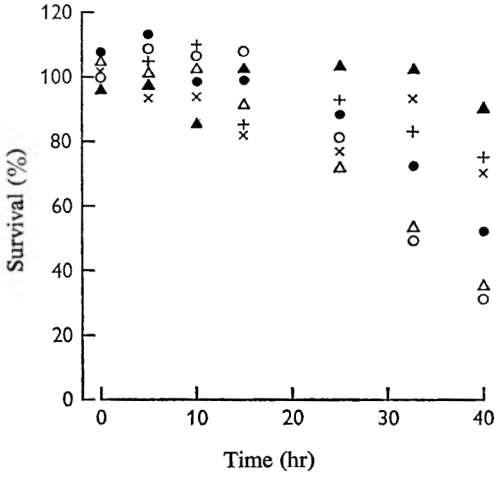

Fig. 1

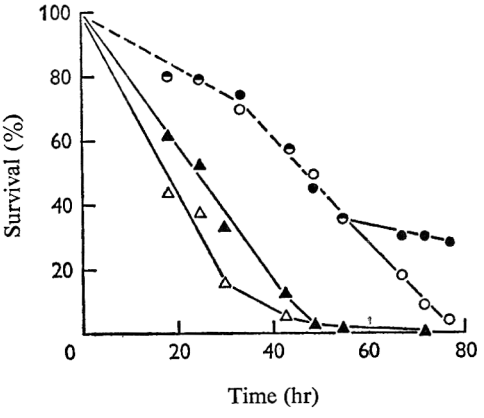

Fig. 2

Fig. 1. Scatter diagram showing oidial viability following incubation in media containing different concentrations of glucose and deGlc. Viability at each point is expressed as % of the control value at zero time. Key: ○, minimal medium containing 10 mM-glucose (= control); △, 10 mM-glucose + 5 mM-deGlc; ×, 10 mM-glucose + 25 mM-deGlc; +, 10 mM-glucose + 50 mM-deGlc; ●, 1·0 mM-glucose + 25 mM-deGlc; ▲, 1·0 mM-glucose + 50 mM-deGlc.

Fig. 2. Curves showing oidial viability following incubation in four fungistatic media. Curve 1 (●) normal minimal medium containing 5 mM-fructose + 0·5 mM-deGlc; curve 2 (○) medium lacking all nitrogen and carbon sources (points showing no clear difference in viability in curves 1 and 2 are plotted as half-closed circles); curve 3 (▲) medium lacking nitrogen sources but containing 5 mM-fructose + 0·5 mM-deGlc; curve 4 (△) medium lacking nitrogen sources but containing 5 mM-fructose.

not to occur on such media. The results given in Fig. 2 show the survival of oidia after incubation in several nutritionally deficient media. There was in general a distinct decrease in viability following incubation in fungistatic media. Though curve 1 of Fig. 2 may suggest that the addition of deGlc to what was essentially the normal medium may have been the cause of the decreased viability, comparison with the other curves shows this was not the case. Any effect on viability in this system was a secondary consequence of the fungistatic action of deGlc and cannot be ascribed to a direct fungicidal action. It is concluded that, at least under the conditions tested, deGlc is not fungicidal towards the coprinus.

Inhibition of extension growth

The rate of extension growth of the coprinus was profoundly affected by deGlc. As Fig. 3 and 4 show, the effective ratio of normal hexose to deGlc depended greatly on the identity of the normal hexose. Growth was decreased by about 50 % on media containing equimolar amounts of glucose and deGlc; a similar degree of inhibition was obtained with fructose + deGlc, but with these compounds in a molar ratio of 100:1.

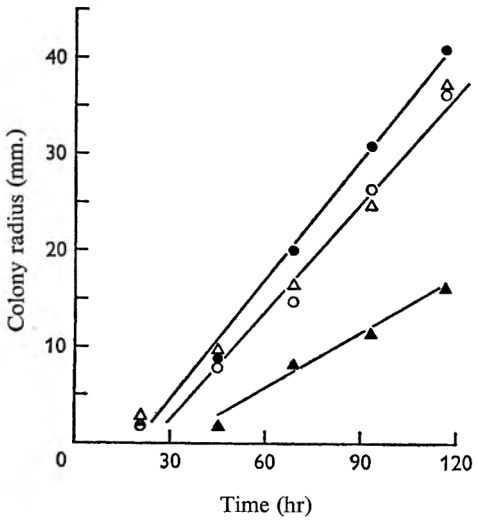

Fig. 3

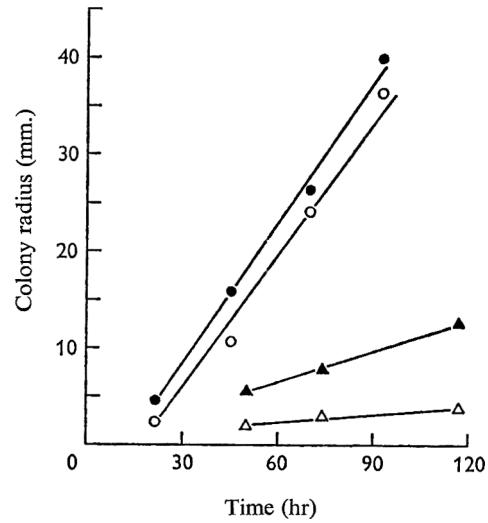

Fig. 4

Fig. 3. Growth rates of colonies on solid media containing: 5 mM-glucose (●); 2·5 mM-glucose (○); 5 mM-glucose + 5 mM-deGlc (▲); 5 mM-glucose + 0·5 mM-deGlc (△).

Fig. 4. Growth rates of colonies on solid media containing: 10 mM-fructose (●); 5 mM-fructose (○); 5 mM-fructose + 0·05 mM-deGlc (▲); 5 mM-fructose + 0·25 mM-deGlc (△).

No growth at all was observed within 120 hr on media containing deGlc alone; concentrations up to 10 mM were tested. Similarly, no growth was obtained on media containing equimolar (5 mM) amounts of fructose and deGlc; 5 mM-fructose + 2·5 mM-deGlc; or 5 mM-fructose + 0·5 mM-deGlc. This fungistatic influence of deGlc towards the coprinus was annulled by adding extra amounts of glucose or fructose; 5 to 10 times as much fructose was required as compared with glucose. This annulment of inhibition could be effected up to the maximum time that inocula were maintained on fungistatic media (120 hr). Similarly, inocula removed from fungistatic media to normal medium, even after 120 hr, grew immediately with normal vigour.

These experiments made it clear that deGlc was not utilized by the coprinus when it was the sole available hexose, and that its fungistatic effects could be completely annulled. The experiments revealed an interesting pattern of sensitivity to deGlc. It appears from the curves in Fig. 3 and 4 that, judging from effective concentrations, the coprinus was at least 100 times more resistant to the effects of deGlc when glucose was present than when fructose was present. Since it is known from oidial survival experiments that colonies appear within 40 hr in media containing deGlc:glucose in

a 50:1 ratio, it is highly likely that this sensitivity differential is very much more pronounced than that indicated here. The problem of this differential sensitivity will be returned to later.

Oxygen uptake

Although deGlc alone did not support growth, oxygen uptake did proceed (Fig. 5); though only at a rate about 25 % of that observed on the same concentration of glucose. The same comparison can be made between growth and O_2 uptake on 5 mM-fructose + 5 mM-deGlc. In general the inhibitory effect of deGlc on O_2 uptake followed fairly closely its effect on growth; the differences were largely a matter of degree. This was also a feature of previous studies; it would appear that the inhibition of a single metabolic process was less dramatic than the cumulative inhibition of the entire

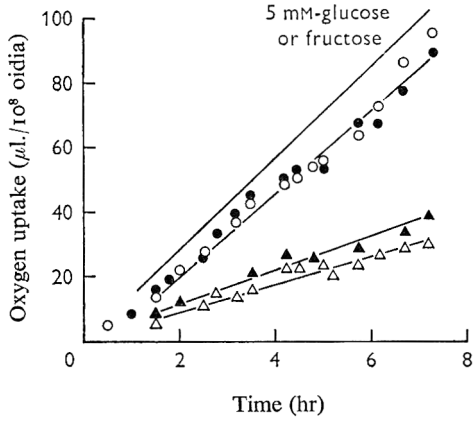

Fig. 5

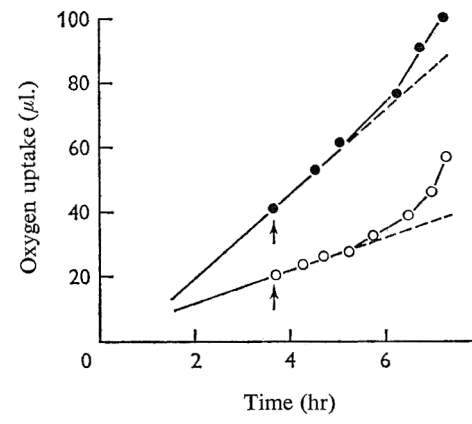

Fig. 6

Fig. 5. Oxygen consumption by spores suspended in liquid media containing: 5 mM-deGlc (△); 5 mM-glucose + 5 mM-deGlc (○); 5 mM-fructose + 5 mM-deGlc (▲); 5 mM-fructose + 0·05 mM-deGlc (●).

Fig. 6. Annulment of the inhibitory effects of deGlc. At the times indicated by the arrows, glucose to a final concentration of 15 mM was tipped from the side-arm into the main compartment of Warburg flasks. Up to that point the spores had been suspended in media containing: 5 mM-glucose + 5 mM-deGlc (●); 5 mM-fructose + 5 mM-deGlc (○).

metabolic complex. Addition of more normal substrate to vessels containing an inhibitory concentration of deGlc annulled that inhibition (Fig. 6); glucose was at least 5 to 10 times more effective than fructose.

The graphs in Fig. 5 show that the differential sensitivity previously referred to applied as much to O_2 uptake as to extension growth. The rate of O_2 uptake measured in experiments involving 5 mM glucose + 5 mM deGlc was very similar to the rate measured in experiments with 5 mM fructose + 0·05 mM deGlc; there was at least a 100-fold difference in sensitivity.

It is clear that both growth and O_2 uptake of the coprinus were much affected by deGlc, and that the inhibition was readily annulled. There was an extreme difference in sensitivity depending on the identity of the normal hexose. Differential sensitivity of this sort has been noted before, but not on so great a scale as observed here. Barban & Schulze (1961) quoted, for human cells, a 5 to 10 times greater sensitivity

with fructose as compared with glucose; from the data of Heredia *et al.* (1964) it appears that *Saccharomyces cerevisiae* was about twice as sensitive to the growth inhibitory effects of deGlc in the presence of fructose. In both of these cases the effects in experiments with mannose and deGlc were virtually identical to those observed with glucose and deGlc. In these two studies, the most closely related in scope to the present one, no growth occurred on equimolar glucose + deGlc; growth of the coprinus was only 50 % inhibited under such conditions. The sensitivity of the coprinus on fructose media was closely similar to the general sensitivity pattern of normal yeast and human cells. On the other hand the coprinus sensitivity on media containing glucose paralleled the sensitivity of resistant sub-lines of HeLa cells (Barban, 1962) and resistant mutants of yeast (Heredia & Sols, 1964). Yet these extremes in the expression of sensitivity have been observed in the same strain of *Coprinus lagopus*. There are many steps in sugar metabolism capable of providing a reasonable explanation for this sensitivity differential. Among these are, for example, competitive inhibition of sugar uptake favouring the aldohexose at the expense of the ketohexose, or a much greater inhibition of fructose phosphorylation than glucose phosphorylation, possibly due to a lesser affinity of hexokinase for fructose. Preliminary experiments suggest that the overall sensitivity difference may well be the cumulative result of several different individual effects.

My thanks are due to Dr D. H. Morgan of the John Innes Institute for supplying me with the *Coprinus lagopus* strain BC9/66 used in this work.

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Lipolytic Activity of Human Cutaneous Bacteria

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SUMMARY

Forty-two strains of *Corynebacterium acnes* were compared with 20 strains of staphylococci and 10 of lipophilic diphtheroids for their lipolytic action on 14 substrates and for Tween 80 hydrolysis. All strains were isolated from normal human skin. One lipophilic diphtheroid and 33 *C. acnes* strains hydrolysed tributyrin. Forty-nine bacillary strains hydrolysed Tween 80. None of the other substrates was degraded. *C. xerosis* ATCC 373 and *Propionibacterium acnes* ATCC 11828 acted on tributyrin. *P. acnes* also hydrolysed Tween 80. The staphylococci did not act on Tween 80 but attacked tributyrin, triolein, trilinolenin, trilinolein and oil emulsions. Some cocci acted on tricaprylin, tricaprin and trilaurin; no lipolysis by this group was observed of trimyristin, tripalmitin or tristearin. Pancreatic lipase, tested by the penicylinder diffusion method for comparison, was active on the same substrates, as were the cocci. The addition of glucose, lactate and urea to media did not inhibit lipolysis by the cocci. Sodium oleate inhibited pancreatic lipase action on triolein and cottonseed oil but not on the hydrolysis of tributyrin. Coccal lipase action was not inhibited by oleate. All 74 test strains were uniformly inhibited by neomycin 5–10 μg./ml. Gram-positive cocci rather than *C. acnes* appear primarily responsible for bacterial lipase activity on human skin.

INTRODUCTION

Previous studies indicate that the predominant microbial flora of normal human skin consists of only three groups of bacteria. These include the staphylococci and micrococci, facultative diphtheroids, many of which are lipophilic, and *Corynebacterium acnes* (Marples, 1965). Indirect evidence suggests that cutaneous bacteria are partially responsible for lipase action on skin, causing the release of fatty acids from the hydrolysis of sebum or triglycerides (Strauss & Mescon, 1959). Staphylococci and micrococci produce lipases and many strains hydrolyse polyoxyethylene sorbitan compounds such as Tween 80 (Baird-Parker, 1963). *C. acnes* hydrolyses Tween 80 (Brzin, 1965) and has been reported to act on tributyrin (Puhvel, 1967). Little is known about *C. acnes* action on other lipids or the possible effects of lipophilic diphtheroids on tributyrin or similar substrates. To gain more information about the lipolytic activity of the skin flora, studies were made to compare lipolysis by *C. acnes* strains with other human skin bacteria.

METHODS

Organisms. Cotton-wool swabs were moistened in sterile saline and rubbed over an area of about 4 × 4 cm. of the faces or forearms of normal human adults; these swabs were discarded. A second swab was rubbed over the same area, placed in 10 ml. sterile saline, and mixed for 30 sec. with an automatic tube shaker. The excess saline was pressed out of the swab into the tube and the swab removed. This saline suspension was used for plating experiments and streaking on the following media: Mannitol salt and Staphylococcus 110 agars (Baltimore Biological Laboratories), Veillonella agar (Difco) and 5% sheep blood agar with heart infusion base (Difco). One set of these plates was incubated aerobically at 35° for 5 days. A second set including only Veillonella and blood agars was incubated anaerobically in Brewer jars with Gaspaks (BBL) and placed at 35° for 5–7 days. Gram-positive cocci were isolated from mannitol salt or 110 agars. Strains were transferred in brain heart infusion broth (BHI), re-streaked on either selective medium and re-isolated. Cocci were characterized by using the methods of Baird-Parker (1963). Facultative diphtheroids were isolated from blood agar. Isolates of this group were identified by Gram reaction and provisionally as lipophilic strains by their greater growth in BHI broth containing sodium oleate or Tween 80 (Pollock, Wainwright & Mansion, 1949). Efforts to classify this group were attempted by using standard procedures (*Manual of Microbiological Methods*, 1957). *Corynebacterium acnes* strains were isolated from anaerobically incubated plates. They were identified by using current information on the diagnostic characteristics of this organism (Prevot, 1966) and confirmed by agglutination by using the methods and antisera for *C. acnes* of the National Communicable Disease Center, Atlanta, Georgia, U.S.A. Cocci and diphtheroids were maintained on slopes of heart infusion agar containing 0·1% yeast extract, 0·2% glucose and 0·2% Tween 80. *C. acnes* strains were maintained in thioglycollate medium (BBL). *Propionibacterium acnes* ATCC 11828 and *C. xerosis* ATCC 373 were included in the study for comparison.

Measurement of lipolysis. Lipolytic activity was detected by using Spirit blue agar (Difco). Triglycerides (Sigma Chem. Co., St Louis, Mo.), corn oil, olive oil, cholesterol and cottonseed oil (Proflo oil, Traders Oil Mill Co., Fort Worth, Texas) were prepared as 20% (w/v) emulsions with a 2·5% sterile solution of gum acacia. Oil emulsions were autoclaved at 118° for 10 min; triglyceride emulsions were sterilized by flowing steam. Substrates were incorporated into the medium in final concentrations of 0·6% (v/v). Cocci and diphtheroid plates were incubated for 2–5 days. *Corynebacterium acnes* plates were incubated in Brewer jars for 7–8 days. Lipolysis was indicated by clearing of the emulsions. Strains which were negative or weakly positive were examined under a dissecting microscope for evidence of lysis under or at the edge of the colonies.

A crude pancreatic lipase (Nutritional Biochem. Corp., Cleveland, Ohio) was prepared as a 1% aqueous suspension (pH 8·0) and tested for its activity on the test substrates by filling penicylinders placed in the centre of each plate, and incubated overnight at 35 degrees. On removal of the cylinders, areas of lipolysis were usually 8–10 mm. in diameter.

In later experiments, certain compounds were incorporated into Spirit blue agar to measure their effects on the lipase activity of the bacterial isolates. These compounds

included glucose, sodium lactate, sodium oleate and urea which were sterilized separately by filtration and added to melted media at 46°.

Strains were tested for neomycin sensitivity in the medium of Schultz-Haudt & Scherp (1956). Neomycin solutions were sterilized by filtration. Growth of the strains was measured in a Spectronic 20 colorimeter (Bausch and Lomb). Tween 80 hydrolysis was measured by the method of Wayne (1962).

RESULTS

The Gram-positive cocci were classified as group 2 staphylococci. The lipophilic diphtheroids were Gram-positive catalase-producing bacilli which consistently grew in palisade forms. Glucose and sodium pyruvate were fermented; maltose, sucrose, fructose, mannitol and lactose were not fermented. These strains were also negative in the following tests: urease and indole production, nitrate reduction, gelatin liquefaction, action on litmus milk and motility. Growth occurred in broths containing 6·5% and 7·5% NaCl and feebly in 15% NaCl broth. One strain produced a brown pigment; the other strains were non-pigmented. These strains were tentatively considered related to the Corynebacteriaceae because of their morphology and salt tolerance (*Bergey's Manual*, 1957).

Table 1. *Lipolytic activity of selected strains of the predominant human cutaneous flora*

	Group tested			
Substrate	Staphylococci	Lipophilic diphtheroids	*Corynebacterium acnes*	Pancreatic lipase
	Number of positive strains			
Tributyrin	20	1	33	+
Tricaprylin	10	0	0	+
Tricaprin	10	0	0	+
Trilaurin	10	0	0	+
Trimyristin	0	0	0	−
Tripalmitin	0	0	0	−
Tristearin	0	0	0	−
Triolein	20	0	0	+
Trilinolein	19	0	0	+
Trilinolenin	19	0	0	+
Olive oil	19	0	0	+
Cholesterol	0	0	0	−
Cottonseed oil (Proflo)	19	0	0	+
Corn oil	19	0	0	+
Tween 80 hydrolysis	0	9	40	nt*
Total strains tested	20	10	42	

* nt, not tested.

Forty-two strains of *Corynebacterium acnes* were compared with 20 staphylococci and 10 lipophilic diphtheroids for their lipolytic action on 14 lipid substrates and for Tween 80 hydrolysis (Table 1). One lipophilic diphtheroid and 33 *C. acnes* strains hydrolysed tributyrin and 40 strains hydrolysed Tween 80. None of the other substrates was attacked by these two groups. *C. xerosis* ATCC 373 and *Propioni-*

bacterium acnes ATCC 11828 acted on tributyrin. *Propionibacterium acnes* hydrolysed Tween 80. The Gram-positive cocci acted on tributyrin, triolein, trilinolein, trilinolenin and the oil emulsions; only half of the staphylococci attacked tricaprylin, tricaprin and trilaurin and no action was observed by this group on trimyristin, tripalmitin or tristearin. Repeated tests with these three compounds at 0·1 % concentrations were negative. Tween 80 was not hydrolysed by the cocci. Pancreatic lipase was active on the same substrates as the lipase-producing cocci.

Lipolytic activity of the cutaneous cocci was also shown by enumerating this group in plating experiments (Table 2). Colony counts of lipolytic bacteria on Spirit blue agar were almost identical to those on mannitol salt agar. Lipolytic colonies from both Spirit blue media showed only Gram-positive cocci; similar results were obtained incubating the plates anaerobically. Lipophilic diphtheroids and *Corynebacterium acnes* were not routinely found among the nonlipolytic colonies developing on Spirit blue media.

Table 2. *Enumeration of the major lipolytic bacteria on human skin*

Medium	Colony counts/sample*
Total	126,000
Mannitol salt agar	85,000
Spirit Blue agar	
+ Tributyrin	85,000
+ Cottonseed oil	83,000

* Counts based on facial swab of 16 cm.^{2} area diluted in sterile saline. Plates were incubated aerobically 5 days at 35°. Total count medium consisted of heart infusion agar with 0·2% glucose, 0·2% Tween 80 and 0·1% yeast extract. Only lipase forming colonies were counted on Spirit Blue agar.

The cocci and pancreatic lipase were retested for lipase action on tributyrin, triolein and cottonseed oil. The following compounds were separately added to each medium: glucose, 2 mg./ml.; sodium lactate, 1 mg./ml.; urea, 0·5 mg./ml.; sodium oleate, 304 μg./ml. (this is equal to a final concentration of 1 μM). Sodium oleate inhibited pancreatic lipase action on triolein and cottonseed oil but not on tributyrin. The other compounds were not inhibitory to pancreatic lipase. The lipolytic action of the cocci was not inhibited by any of the added compounds.

The incorporation of neomycin sulphate 5–10 μg./ml. to broth or agar media inhibited the growth of all strains.

DISCUSSION

Gram-positive cocci appear to be the only major cutaneous bacteria with appreciable lipase activity. These results provide evidence that *Corynebacterium acnes* is not one of the predominant lipolytic bacteria of skin. Inhibition of bacterial lipase in tissue sections treated with neomycin spray (Strauss & Mescon, 1959) is more likely to be due to inhibition of coccal growth and not *C. acnes* growth or activity.

Glucose has been reported to suppress microbial lipase formation (Smith & Alfords, 1966) but the glucose concentration used in our test medium did not prevent the action of the lipolytic cocci. Lactate and urea, which are excreted in large quantities by the skin (Marples, 1965) were also inactive as lipase inhibitors. Certain lipases are known to be inhibited by a concentration of μM-oleate (Smith & Alford, 1966).

This was observed by using pancreatic lipase with a cylinder diffusion method. The accumulation of oleic acid on skin would not appear to inhibit staphylococcal lipase activity. The continued recognition of new lipids secreted by skin (Kellum, 1967*a*, *b*) may indicate that many more substrates are available for bacteria to act upon. *Corynebacterium acnes*, which resides primarily in the pilosebaceous units of the skin (Marples, 1965) might act differently in response to certain yet unidentified lipids. The C_{18} unsaturated triglycerides were hydrolysed while failure of the cocci and the pancreatic lipase to act upon trimyristin, tripalmitin and tristearin may be a function of chain length and saturation of the compounds. Variations in qualitative determinations of lipase activity has been reported (Muys & Willemse, 1965). Spirit blue agar was generally satisfactory for this comparative study.

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The Effect of Cerebroside and Other Lipids on the Fixation of Tetanus Toxin by Gangliosides

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SUMMARY

Water-insoluble complexes of ganglioside with cerebroside fixed tetanus toxin at low concentrations (a few LD 50/ml.) of toxin. A complex containing 25 % ganglioside with cerebroside was 50 times better at fixing toxin than complexes containing either 2 % or 50 % ganglioside. A complex containing 25 % of a mixture of the gangliosides G_{III} and G_{IV} was 12 times better at fixing toxin than a similar complex with gangliosides G_I and G_{II}. Complexes of ganglioside with sphingomyelin and lecithin fixed toxin to a slight extent, while complexes with tripalmitin and cholesterol did not fix toxin. The complex of cerebroside and ganglioside, containing 25 % ganglioside, did not fix strychnine, serotonin, botulinum toxin or plasma albumin.

INTRODUCTION

Whole brain homogenate fixes tetanus toxin (Wassermann & Takaki, 1898). The only purified components of nervous tissue which have been found to fix toxin are the gangliosides. This fixation has been demonstrated in the analytical ultracentrifuge where, at high concentrations of toxin (5 mg./ml., 30 million LD 50/ml.), ganglioside will fix up to 20 times its own weight of toxin (van Heyningen, 1963). The gangliosides are water-soluble but can be made insoluble by complexing them with other substances, such as cerebrosides, which are themselves insoluble in water. The cerebrosides do not fix tetanus toxin but their insoluble complexes with gangliosides do, and these can be used to study toxin fixation at low concentrations (a few LD 50/ml.) of toxin (van Heyningen, 1959*a*, *b*; van Heyningen & Miller, 1961). However, when the ability of nervous tissue to combine with toxin is compared with that of a complex containing equal parts of ganglioside and cerebroside, or with 'protagon' (a crude cerebroside preparation containing about 2·5 % ganglioside; see van Heyningen, 1959*a*, *b*), the toxin-fixing capacity apparently decreases with increasing ganglioside content of the preparations (Table 1). This would appear either to contradict the suggestion that the ability of nervous tissue to fix tetanus toxin is due to its content of ganglioside, or to suggest that some other component of nervous tissue (present also in 'protagon') takes part in the fixation of tetanus toxin by nervous tissue without necessarily being able to fix toxin by itself. In the present work, the fixation of tetanus toxin by complexes of gangliosides with water-insoluble lipids has been further investigated. A preliminary report of the conclusions of some of the work in this paper has appeared elsewhere (Mellanby & van Heyingen, 1965).

METHODS

Tetanus toxin was the preparation TD 464 D kindly supplied by Dr R. O. Thomson of the Wellcome Research Laboratories; it contained 40% protein of which 75% was toxin (see van Heyningen, 1959*a*, *b*). Dilutions of the toxin were made in 0·1 M-phosphate buffer (pH 7·0) containing 0·2% gelatin. Mixed gangliosides were prepared by the method described by Mellanby, Pope & Ambache (1968). Crude cerebroside

Table 1. *Tetanus toxin fixing capacities of various preparations containing ganglioside*

Preparation	Ganglioside (%)	mg. required to fix 10 LD 50 of toxin		Relative toxin-fixing capacity per unit ganglioside
		Total weight	Ganglioside	
Fresh guinea-pig brain homogenate	0·5	0·25	0·00125	800
'Protagon'	2·5	0·40	0·01	100
Ganglioside + cerebroside complex	50	2	1	1

(containing 0·75% ganglioside) was prepared by the method of Klenk & Leupold (1944). Samples of highly purified cerebroside and sphingomyelin (containing no detectable ganglioside) were kindly provided by Professor H. E. Carter of the University of Illinois. Lecithin was obtained from British Drug Houses Ltd. (from egg: 95–100%). The gangliosides G_I, G_{II}, G_{III} and G_{IV} were kindly provided by the late Professor Richard Kuhn of Heidelberg. Total lipids of brain were prepared by homogenizing fresh guinea-pig brain with 30 volumes of chloroform + methanol (2 + 1, v/v) and filtering off the residue. The filtrate was diluted one in four in the same solvent. This solution, representing 8 mg. fresh brain/ml., contained 0·8 mg. dry weight (i.e. total lipids)/ml. Strychnine and serotonin were obtained from British Drug Houses Ltd.

Sialic acid was estimated by the resorcinol method of Svennerholm (1957). Where the determination was to be made on mixtures of lipids, the gangliosides were first partially purified by adsorption on silicic acid followed by elution with methanol. The procedure used was adapted from a method devised by McCluer, Coram & Lee (1962). An appropriate amount of the lipid extract in which the ganglioside was to be measured was evaporated to dryness and the lipids dissolved in 1 ml. methanol followed by 15 ml. chloroform. Silicic acid (1·5 g.; previously washed in absolute methanol, 2 ml./g., and dried overnight at 70°) was weighed into a clean dry fritted glass funnel (porosity 2) and packed by gentle tapping. The silicic acid was wetted with 2–3 ml. chloroform + methanol (15 + 1, v/v) and the lipid solution was applied to it. The solvent was then forced through under positive pressure (rubber bulb) until the solvent was just level with the top of the silicic acid. The silicic acid was then washed in the same manner with 5 ml. chloroform + methanol (9 + 1, v/v) followed by 5 ml. chloroform + methanol (3 + 1, v/v) and the washings discarded. The gangliosides were eluted from the column with two 5 ml. portions of absolute methanol, the second portion of methanol being forced through until the silicic acid was dry. The methanol eluate was evaporated to dryness in a stream of air in a water bath at 60° and the residue was suspended in 2 ml. water (for the determination of sialic acid).

Complexes of ganglioside with water-insoluble lipids were prepared by dissolving the two together in organic solvent, mixing, evaporating off the organic solvent and resuspending the residue in hot water (van Heyningen, 1963). When it was desired to add a constant amount of total brain lipid to decreasing amounts of ganglioside + cerebroside complex, the following procedure was used. The appropriate amounts of ganglioside and cerebroside were dissolved in chloroform + methanol (1 + 1, v/v) and serial 2-fold dilutions of this solution were made with the same solvent. The appropriate volume of the chloroform + methanol extract of brain was then added to each of these dilutions, the solvent evaporated off, and the residues suspended in appropriate constant volumes of water.

Toxin-fixing capacity was measured by adding constant volumes of toxin solution (44 LD 50/ml.) to equal volumes of suspensions containing decreasing concentrations of nervous tissue or lipid complex, standing for 10 min., centrifuging at 20,000 rev./min. for 15 min. (40·2 head in Spinco Model L) and injecting 0·5 ml. volumes of the supernatant fluids intra-muscularly into pairs of mice. In this way the least amount of suspension required to fix 10 LD 50 doses of toxin could be determined (van Heyningen, 1959*b*).

RESULTS

To see whether a lipid component of brain was responsible for the superior toxin-fixing ability per unit ganglioside of a brain homogenate compared with a cerebroside + ganglioside complex (50 % ganglioside) the effect of the addition of the total lipids of a chloroform + methanol (2 + 1, v/v) extract of brain on the toxin-fixing capacity of the complex was tested. The results of these tests are given in Table 2. It can be seen that the total brain lipids had comparatively low toxin-fixing capacity, but the addition of 0·8 mg. of these lipids to cerebroside + ganglioside (containing 50 % ganglioside) increased the toxin-fixing capacity of the complex more than 90-fold. Decreasing the ganglioside content of a complex to 10 % had a similar effect. However, addition of 0·8 mg. total brain lipids to this complex then decreased its toxin-fixing capacity 4-fold. A possible explanation of these apparently contradictory findings on the effect of brain extract on toxin fixation by cerebroside + ganglioside complexes was that the substance in brain extract enhancing toxin-fixation was cerebroside itself

Table 2. *The effect of total brain lipids on the fixation of tetanus toxin by cerebroside: ganglioside complexes*

Lipids	mg. required to fix 10 LD 50 toxin
Pure cerebroside	5·0
Total brain lipids	1·6
Crude cerebroside (containing 0·76 % ganglioside)	0·96
Crude cerebroside + 0·8 mg. total brain lipids	0·85
Crude cerebroside: ganglioside (containing 50 % ganglioside)	1·89
Crude cerebroside: ganglioside (containing 50 % ganglioside) + 0·8 mg. total brain lipids	0·02
Crude cerebroside: ganglioside (containing 10 % ganglioside	0·02
Crude cerebroside: ganglioside (containing 10 % ganglioside) + 0·8 mg. total brain lipids	0·08

and that there is an optimal proportion of cerebroside in the complex—more than 50% but not much different from 90%. Figure 1 shows the results of an experiment in which this possibility was tested. Complexes of ganglioside and cerebroside containing from 2% to 50% ganglioside were prepared and the weights of such complexes required to fix 10 LD50 doses of tetanus toxin were determined. With a complex containing 25% ganglioside only one-fiftieth of the weight of the complex was required as compared with complexes containing either 50% or 2% gangliosides.

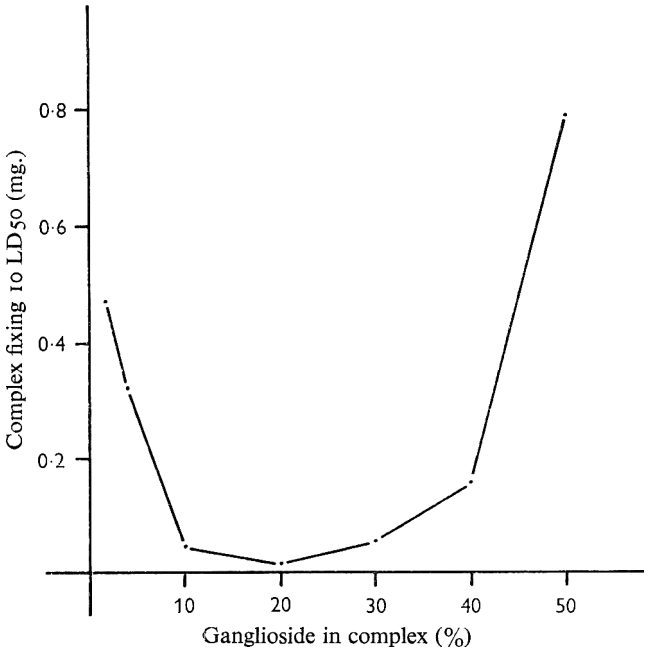

Fig. 1. The effect of the proportion of ganglioside in a cerebroside + ganglioside complex on the toxin-fixing capacity of the complex.

Table 3. *The toxin-fixing capacity of fresh guinea-pig brain and of a cerebroside: ganglioside complex, compared per unit dry weight and per unit ganglioside*

	Weight needed to fix 10 LD50 of toxin				Ratio of ganglioside in complex to ganglioside in brain needed to fix 10 LD50 of toxin (col. 5/col. 4)
	mg. total weight		mg. ganglioside		
Toxin sample	Brain	Complex*	Brain	Complex*	
1	0·59	0·07	0·002	0·02	10
2	0·38	0·03	0·0019	0·008	4
3	0·14	0·01	0·0007	0·0025	4
4	0·09	0·008	0·00045	0·002	4
5	0·6	0·1	0·003	0·024	8

* Cerebroside:ganglioside complex containing 25% ganglioside.

In Table 3 the results of five experiments are given in which the toxin-fixing capacity of brain homogenate and of the cerebroside + ganglioside complex containing 25% ganglioside are compared. In the fourth and fifth column of the table, the toxin-fixing

capacity per unit of ganglioside is shown, and in the last column the ratio of these values is given. Although a 6-fold variability in the toxin-fixing capacity of brain and of the complex was found from experiment to experiment, the toxin-fixing capacity of brain expressed per unit ganglioside was consistently higher (4- to 10-fold) than that of the cerebroside + ganglioside complex.

Complexes of ganglioside with lipid components of brain other than cerebroside were also tested for their ability to fix tetanus toxin. Table 4 compares the ability to fix toxin of complexes containing 25% ganglioside with cerebroside, sphingomyelin, lecithin, cholesterol or tripalmitin. The complex with cerebroside was the most effective in toxin fixation, but the complexes with sphingomyelin and lecithin also had a little toxin-fixing capacity; the cholesterol and tripalmitin complexes did not fix toxin. The toxin-fixing capacity of ganglioside + sphingomyelin complexes was, like that of ganglioside + cerebroside complexes, greater when the proportion of ganglioside in the complex was 25%, rather than 2% or 50%.

Table 4. *A comparison of the toxin-fixing capacity of complexes (containing 25% ganglioside) of ganglioside with different lipids*

Second lipid	mg. complex required to fix 10 LD50
Cerebroside	0·07
Cholesterol	> 8
Sphingomyelin	2·0
Lecithin	5·4
Tripalmitin	> 8

These results suggested that the receptor substance with which tetanus toxin combines, when it is fixed by nervous tissue, might include a complex of ganglioside and a water-insoluble component, probably cerebroside, containing about 25% of ganglioside. The question arose as to whether the combination of tetanus toxin at low toxin concentration (20 LD50/ml.) with cerebroside + ganglioside complexes was an aspect of the same phenomenon as the fixation demonstrated in the ultracentrifuge at high toxin concentration (5 mg. toxin/ml.; 30 million LD50/ml.). It had been shown in the ultracentrifuge that the ability of pure gangliosides to fix toxin depended upon the number and position of the sialic acid residues (van Heyningen, 1963). Complexes with cerebroside were therefore prepared containing 25% either of a mixture of gangliosides G_I and G_{II}, which were comparatively poor toxin-fixers in the ultracentrifuge, or of a mixture of gangliosides G_{III} and G_{IV}, which were six times better. Table 5 shows that when their abilities to fix toxin at low toxin concentration were compared, the $G_{III} + G_{IV}$ complex was twelve times as efficient as $G_I + G_{II}$.

It had also been shown previously, in the ultracentrifuge, that ganglioside alone would fix strychnine and serotonin (5-hydroxytryptamine) (van Heyningen, 1963). In contrast, however, it was found in the present work that a cerebroside + ganglioside complex which fixed tetanus toxin would not fix these substances. Like ganglioside alone, the complex would not fix botulinum toxin or plasma albumin.

Table 5. *Fixation of tetanus toxin by different gangliosides and by their complexes (containing 25% ganglioside) with cerebroside*

Ganglioside	Oligosaccharide moiety	mg. toxin fixed/ mg. ganglioside*	mg. complex needed to fix 10 LD50 of toxin
G_I	Galactose | Galactosamine | Sialic acid—Galactose | Glucose	2·6	0·006
G_{II}	Sialic acid—Galactose | Galactosamine | Sialic acid—Galactose | Glucose	3·6	
G_{III}	Galactose | Galactosamine | Sialic acid—Galactose | | Sialic acid Glucose	19·3	0·0005
G_{IV}	Sialic acid—Galactose | Galactosamine | Sialic acid—Galactose | | Sialic acid Glucose	19·0	

* Measured in the analytical ultracentrifuge; van Heyningen, 1963.

DISCUSSION

It has been found that the optimal proportion of ganglioside for toxin fixation in a ganglioside + cerebroside complex is about 25% ganglioside. The major part of both ganglioside and toxin-fixing capacity resides in the grey matter of brain. The proportions of ganglioside and cerebroside in grey matter are about equal. Thus if the receptor in nervous tissue for tetanus toxin includes such a complex of ganglioside and cerebroside (containing 25% ganglioside), only about one quarter of the ganglioside in grey matter can be involved.

Table 2 showed that it was not possible to account for more than 10–25% of the toxin-fixing capacity of brain per unit ganglioside on the assumption that the receptor for toxin is a complex of cerebroside and mixed gangliosides such as were used here. However, these gangliosides were extracted from dried brain with hot chloroform + methanol, (2 + 1, v/v) and in these extracts the 'slow' gangliosides (G_{III}, G_{IV}) comprised only about 10% of the total gangliosides. This is about a quarter of their proportion in the gangliosides in fresh brain (Suzuki, 1965). It has been shown in the

present work that only about one-twelfth as much of a complex of cerebroside with G_{III} and G_{IV} gangliosides is needed to fix 10 LD50 doses of tetanus toxin as is needed of a complex with G_I and G_{II} gangliosides. Hence, if a ganglioside + cerebroside complex were used in which the ganglioside contained the same proportion of G_{III} and G_{IV} as is present in brain, the toxin-fixing capacity per unit of ganglioside of the complex would be closer to that of brain. It is possible that the receptor substance for tetanus toxin in brain includes a cerebroside + ganglioside complex with other, non-lipid, constituents (perhaps histones; see van Heyningen, 1963).

It was previously shown that a major part of the toxin-fixing capacity of brain resides in the nerve endings (Mellanby, van Heyningen & Whittaker, 1965) and within the nerve endings, it is the membranes rather than the mitochondria or the synaptic vesicles which fix the toxin (Mellanby & Whittaker, 1968). It is possible that within the membrane fraction there is the optimal cerebroside + ganglioside ratio; but this need not be the case since there might of course only be small receptive areas on the membrane where the ratio is optimal, the ratio of cerebroside to ganglioside in the whole fraction not being optimal for fixation. For this reason it did not seem likely to be particularly fruitful to investigate the cerebroside:ganglioside ratios of subcellular fractions of the brain.

The fixation of tetanus toxin by high concentrations of gangliosides, as demonstrated in the ultracentrifuge, is fairly specific—none of the other enzymes, toxins or inert proteins that were tested were fixed except, to a far lesser extent, tetanus toxoid; but gangliosides also fix strychnine, bruceine, thebaine and serotonin under these conditions (van Heyningen, 1963). If the fixation of tetanus toxin by mixed gangliosides were relevant to its mode of action, the fixation of these drugs, the first three of which have a physiological action comparable to that of tetanus toxin, might also be relevant to the biochemical action of tetanus toxin. However, it has been shown in the present work that strychnine and serotonin were not fixed by the cerebroside + ganglioside complex. It can therefore be seen that the fixation of tetanus toxin by cerebroside + ganglioside is more specific for tetanus toxin than is the fixation by ganglioside alone. The complex is therefore perhaps more likely than ganglioside alone to be included in the receptor for tetanus toxin in nervous tissue. The specificity of the combination between ganglioside and tetanus toxin with respect to the structure of the ganglioside is retained when the gangliosides are combined with cerebroside; the gangliosides which contain two sialic acid residues joined to each other are much better in fixation.

Van Heyningen & Woodman (1963) showed that frog brain has a low toxin-fixing capacity compared with mammalian brain, and that frog brain ganglioside is extractable from the brain with water. The toxin-fixing capacity of frog brain ganglioside does not appear to differ from that of mammalian brain ganglioside. In mammalian brain the ganglioside is not extractable with water, and possibly is more firmly bound in water-insoluble complexes than frog brain ganglioside. This may be relevant to the relatively poor toxin-fixing capacity of frog brain.

Although this work may further have defined the receptor for tetanus toxin in nervous tissue and have shown it to be a more specific receptor for the toxin than ganglioside alone, it has not of course any further elucidated the role (if any) of ganglioside in the lethal action of tetanus toxin.

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The Independence of the Lysolecithinase Activity of Extracts of *Clostridium sordellii* from Their Lethal, Oedema-producing and Haemorrhagic Actions

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SUMMARY

Extracts (ultrasonic treatment) of *Clostridium sordellii* contained a lysolecithinase, which was activated by ether, calcium ions and ammonium sulphate and inhibited by EDTA, magnesium and manganese. The enzyme was not responsible for the lethality, oedema-producing or haemorrhagic actions of extracts of the organism.

INTRODUCTION

Extracts of *Clostridium sordellii* are lethal and dermonecrotic (see for example Brooks & Epps, 1959), and intradermal injection produces lesions involving haemorrhagic areas of skin with underlying gelatinous oedema. It is not known to what enzymic action the pathological effects may be ascribed. Lewis & Macfarlane (1953) confirmed the observation of Miles & Miles (1947) that a phospholipase C can be produced by *C. sordellii* (*bifermentans*). Although this enzyme is toxic, it is much less toxic per unit of phospholipase activity than the antigenically similar phospholipase C produced by *C. welchii*. Moreover, in the present work a strain of *C. sordellii* was used which does not produce a phospholipase C and yet culture filtrates and extracts of the organism are nevertheless lethal and dermonecrotic.

During a search for other lipolytic enzymes which might be responsible for the pathological action of *Clostridium sordellii*, it was found that the organism consistently produced a lysolecithinase. Arseculeratne (1965) had produced preliminary evidence, in this laboratory, that such an enzyme might be produced. Since lysolecithin is a more toxic substance than glycerylphosphorylcholine, the product of lysolecithinase activity, one might perhaps not expect a lysolecithinase to have any pathological activity. On the other hand, lysolecithin is probably a component of natural membranes and therefore its breakdown might result in pathological effects. To investigate whether there was any correlation between lysolecithinase activity and the pathological action of *C. sordellii* two approaches were used. Firstly, the organism was grown for 4, 8 and 12 hr and the various activities measured in culture supernatant fluids, in extracts made by ultrasonic treatment and in saline extracts of the bacteria at these three times. It was hoped to see whether there was any correlation, in time of production or extractability, between the activities. Secondly, the enzyme was put through a 6-fold purification step and the pathological activities of the fractions with low and high lysolecithinase specific activities compared.

METHODS

Lysolecithin and lysophosphatidylethanolamine (prepared from lecithin and phosphatidylethanolamine, respectively, by the action of snake venom phospholipase A) and *Ancistrodon piscivorus* venom were obtained from Koch–Light Laboratories Ltd (Colnbrook, Buckinghamshire, England).

The strain of *Clostridium sordellii* used was kindly provided by the Wellcome Research Laboratories. The organism was grown for 4 hr at 37°, in 1 l. flasks or 10 l. bottles, in a medium containing: 30 g. Oxoid Bacteriological Peptone, 7·5 g. Na-β-glycerophosphate and 0·1 ml. of neutralized thiolacetic acid (approximately 0·3 mmoles), in 1 l. water. This medium was inoculated with an inoculum (2 ml.) of a culture grown overnight in cooked meat medium, which had itself been inoculated per 10 ml. with 1 drop of a spore suspension (containing equiv. 100 mg. dry wt/ml.) which had been stored in 50 % (v/v) glycerol in water at −40°. The bacteria were collected by centrifugation and washed once in 1/5 of the original volume of 0·9 % sodium chloride. The equiv. dry weight of organisms in the culture was estimated by measuring the extinction at 600 mμ of a suitably diluted sample. There was a linear relation between equiv. dry weight and E_{600} over a range of readings up to 0·60. A reading of 0·300 on the spectrophotometer corresponded to 0·7 mg. dry wt organism/ml.

The bacteria were centrifuged down (at 10,000*g* for 10 min.), resuspended in water and given ultrasonic treatment for 10 min. in an MSE sonicator (using a power of 100 W). The broken suspension was then centrifuged at 17,000*g* for 10 min.; the supernatant fluid constituted the sonic extract. This was passed through a Seitz bacteriological filter and the protein in the extract precipitated by adding solid ammonium sulphate (707 g./l.), keeping the mixture at 2° and pH 7·5. This ammonium sulphate precipitate contained large amounts of material with an absorption at 260 mμ, which was not separated from the lysolecithinase activity by pretreatment with protamine sulphate and nucleases, precipitation with acid or passing the preparation through G 75 Sephadex.

The purification step adopted gave a 6-fold increase in specific activity. The procedure involved adsorbing the enzyme from a solution (in water) of the ammonium sulphate precipitate of the sonic extract, on to calcium phosphate, by adding (at 4°) 0·1 volume of 0·2 M-Na_2HPO_4 followed by 0·05 volume of M-$CaCl_2$; the mixture was adjusted to pH 7·5 and allowed to stand at 4° for 30 min. The suspension was then centrifuged in the cold at 12,000*g* for 10 min. The precipitate was stirred gently at 2° for 30 min. with ammonium sulphate solution (containing 177 g./l. = 0·25 saturated) at pH 7·5. The suspension was then recentrifuged; the supernatant fluid contained the 6-fold purified enzyme.

Protein was estimated by the technique of Lowry, Rosebrough, Farr & Randall (1951) on samples which had been dialysed for at least 6 hr against running cold water.

Lethality of preparations was investigated by injecting pairs of Swiss albino mice intramuscularly with 2-fold serial dilutions prepared in gelatin buffer (0·1 M-phosphate buffer (pH 7·0) containing 0·2 % gelatin). One LD50 dose was defined as the dose which killed within 72 hr half the mice injected.

A rough assay system was worked out for oedema-producing and haemorrhagic

effects. Comparisons of the activity of different preparations were made by injecting equal amounts of protein, dissolved in 0·1 ml. gelatin buffer (see above), into the shaved flank skin of albino guinea pigs. The skins were examined 4 hr after injection and the degrees of oedema and haemorrhage assessed on an arbitrary relative scale, ([±], +, + +, + + +) from the external and internal post-mortem appearances. The lesions usually appeared as dark-red round haemorrhagic patches up to 10–15 mm. diam., sometimes with a paler area of necrosis in the middle. Where there was much oedema factor, the oedema spread all round the injected side of the animal as a thick gelatinous layer and the skin adhered tightly to the underlying fascia. However. where there was little oedema factor, the oedema remained as a bleb around the site of injection.

Thin-layer chromatography was done on Kieselgel-G (nach Stahl; Shandon). Incubation mixtures of phospholipid and enzyme were extracted with chloroform + methanol (2 + 1, v/v), the lower chloroform layers were evaporated to dryness and the residue was taken up in 1/5 volume or less of chloroform + methanol (2 + 1, v/v). The solutions were loaded on to the plate in streaks (1 cm.) and the plate developed by ascending chromatography in chloroform + methanol + water (60 + 30 + 5, by vol.). The spots were located by spraying the dried plates lightly with sulphuric acid + water (1 + 1, v/v) and heating at 160° for about 30 min.

The action of a sonic extract of *Clostridium sordellii* on purified lysolecithin was demonstrated by comparing the thin-layer chromatograms of (*a*) lysolecithin alone, (*b*) lecithin alone, (*c*) an extract from an incubation mixture of lecithin and the phospholipase A of the venom of *Ancistrodon piscivorus*, and (*d*) an extract from an incubation mixture of lysolecithin and a sonic extract of *C. sordellii*. The chromatogram from (*c*) showed the lecithin spot replaced by a lysolecithin spot and a fast-moving spot corresponding to the free fatty acid liberated by the phospholipase A; in the chromatogram from (*d*) the lysolecithin spot was absent, but a free fatty-acid spot similar to that in (*c*) was present. This showed that the sonic extract of *C. sordellii* contained a lysolecithinase liberating free fatty acid from lysolecithin.

Assay of lysolecithinase activity

The preparations from *Clostridium sordellii* were incubated with lysolecithin (0·25 mg./ml.) in phosphate buffer (0·004 M; pH 7·5) in a final volume of 1 ml. + initially 1 ml. ether, at 30° for 30 min. The reaction was started by adding the enzyme and stopped by placing the mixture in ice and adding 5 ml. of extraction mixture (iso-propylalcohol + *n*-heptane + 2 N-H_2SO_4; 80 + 20 + 1, by vol.). The free fatty acids extracted were then titrated after the method described by Kelley (1965) with N/200 tetra-*n*-butyl ammonium hydroxide in methanol, by using an automatic titrator (Radiometer, Copenhagen; type ABU 1 b; supplied by V. A. Howe, London).

RESULTS

Some properties of the lysolecithinase

When ether was added to the incubation mixture it was found that a constant rate of reaction for at least 30 min. was obtained. In the absence of ether the rate decreased after the first 5 min. There was an optimal value around pH 7·5; for a 15 min. incubation period (in the absence of ether) the optimal temperature was around 30°;

in the presence of ether the activity of the enzyme was decreased by EDTA and magnesium and manganese salts (0·01 M final concentration); and it was completely inhibited (in the absence of ether) by deoxycholate (0·012 M); it was activated by calcium chloride (0·01 M) and by ammonium sulphate (3·5 M). With lysophosphatidylethanolamine as substrate for the enzyme (in the presence of ether) the reaction proceeded at about half the rate of that with lysolecithin.

Effect of time of incubation of Clostridium sordellii on the production of lysolecithinase and other active substances

A comparison was made of the lysolecithinase activity, the lethality (in mice), haemorrhage-producing and oedema-producing actions (in guinea-pig skin), haemolytic activity, and proteolytic activity of supernatant fluids and extracts from the *C. sordellii* incubated and harvested at 4, 8 or 12 hr. Table 1 shows that the lyso-

Table 1. *Production of various biological activities by a culture of Clostridium sordellii*

Samples of the culture were taken after incubation for 4, 8 and 12 hr. Activities were measured in the culture supernatant fluid and in sonic extracts of the organisms. The results from two similar experiments are given. The activities are given per ml. of original culture.

	Time of incubation					
	4 hr		8 hr		12 hr	
	Supernatant fluid	Sonic extract	Supernatant fluid	Sonic extract	Supernatant fluid	Sonic extract
LD50	80	<10,000	1,600	4,800	1,600	9,600
	8	3,840	1,920	960	2,560	1,920
Lysolecithinase	> 0·1	0·45	> 0·1	0·43	0·3	0·40
	> 0·1	2·8	> 0·1	1·4	> 0·1	2·2
Haemorrhagic activity	0	+++	++	++	++	++
	0	+++	++	++	++	++
Oedema activity	0	++	±	+	±	±
	0	0	0	++	+	+
Haemolysis units	7·2	0·02	4·1	0·02	2·8	0·02
	10	0·08	6·5	0·08	5·8	0·10
Protease	0·61	0·03	0·81	0·03	0·65	0·03
	0·72	0·045	0·96	0·045	1·02	0·04

lecithinase activity remained within the organisms for at least 8 hr and was still mainly intracellular after 12 hr. On the other hand, very weak proteolytic and haemolytic activities, almost paralleling each other, were found almost exclusively in the culture supernatant fluid. Oedema-producing activity was also unrelated to lysolecithinase activity, since it was decreasing between 4 and 12 hr while the lysolecithinase remained approximately constant over this period. From this kind of experiment it was not possible to say whether or not there was any relation between lysolecithinase activity and the haemorrhagic (guinea-pig skin) activity.

Pathological activities of preparations differing in their lysolecithinase content

In Table 2 the pathological activities of two preparations whose lysolecithinase activity differed about 7-fold are compared. The two preparations were the super-

natant fluid (CaSF) after most of the lysolecithinase activity of a sonic extract of *Clostridium sordellii* had been adsorbed on calcium phosphate, and the material, enriched in lysolecithinase activity, eluted from the calcium phosphate with 0·25 saturated ammonium sulphate (0·25 SF). It can be seen that the haemorrhagic

Table 2. *Pathological activities of preparations from Clostridium sordellii differing in their lysolecithinase content*

See text for method of preparation.

	Lysolecithinase activity (units/mg. protein)	Lethality (LD50/mg. protein)	Oedema-producing activity (0·01 mg. protein)	Haemorrhagic activity (0·01 mg. protein)
Sonic extract of organisms	0·42	133,000	++	++
Supernatant after calcium phosphate adsorption (calcium SF)	0·32	400,000	(+)	++
Material eluted from calcium phosphate with ammonium sulphate solution (0·25 SF)	2·45	133,000	+	++

activity of the two preparations was the same; hence the lysolecithinase was independent of the haemorrhagic activity. It was confirmed that lysolecithinase was independent of the lethality. Table 2 also further illustrates (as can be seen in Table 1) that the oedema factor was not chiefly responsible for the lethal action of preparations from *C. sordellii*, and that the oedema and haemorrhagic factors could be independent. It can be concluded that the lysolecithinase of *C. sordellii* is not responsible for the lethal, oedema-producing or haemorrhagic activity of extracts of this organism.

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Intraconidial Conidia in the Spray Mutant of *Neurospora crassa*

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SUMMARY

The 'spray' mutant of *Neurospora crassa* responded differently from the wild type when grown in conidiogenic (C) and mycelial (M) media. In both media spray produced an unpigmented non-conidiating form of growth, whereas the wild type formed pure mycelial cultures on M-medium and conidiating growth on C medium. Analysis of the fine structure of spray revealed that the modal width of mitochondria of C- and M-media grown cultures was virtually identical. Since it is known that C-cultures of wild-type *N. crassa* possess swollen mitochondria, a relationship was suggested between mitochondrial swelling and the metabolic mechanisms which govern conidial differentiation. Spray also varied from the wild type in cell-wall thickness. Intraconidial conidia were observed in the spray mutant. These internal conidia always contained normal cytoplasm, while that of the host cell consisted of degenerate components. Intra-conidial conidia might arise as a result of proliferation of the septal wall.

INTRODUCTION

The changes in fine structure accompanying the process of conidiation in Neurospora have been examined by several investigators. Weiss & Turian (1966) reported that conidial cultures (C-cultures) of wild-type *Neurospora crassa* possessed mitochondria which were considerably more swollen than those of mycelial cultures (M-cultures). These morphologically distinct forms were obtained by growing wild-type *N. crassa* on C-medium, which favours conidiation, or on M-medium, which gives mycelial cultures (Turian, 1964). The study of conidiation has been extended to various morphological mutants of Neurospora. Lowry, Durkee & Sussman (1967) described the events taking place during microconidial formation in the 'peach-fluffy' mutant, and Turian, Oulevey & Tissot (1967) and Oulevey-Matikian & Turian (1968) observed the fine structure of the 'fluffy' and 'amyc' mutants. The present paper is concerned with the detailed structural analysis of the spray morphological mutant. Its purpose was twofold. First, since spray differed from the wild type in that it did not respond to the conidiating effect of the C-medium, and therefore C- and M-cultures were identical, an electron-microscope study was made to determine whether or not the swelling of the mitochondria was related to conidial differentiation or was merely due to the composition of the medium. Secondly, with light microscopy, this mutant was seen to possess a wide variety of sizes and shapes of conidia. Some conidia resembled those of wild-type Neurospora whereas others were more elongated.

A large percentage of mature conidia tended to remain attached to neighbouring cells, and certain conidia were found which served as hosts to internal cells which were considered to be intraconidial conidia.

METHODS

Organisms. The spray mutant (68*A*) was obtained from the Fungal Genetics Stock Center, Dartmouth College, Hanover, New Hampshire, U.S.A., and was originally isolated by D. D. Perkins.

Growth conditions. Conidia were obtained by growing the organism at 25° on Westergaard and Mitchell solid minimal medium which was modified with respect to potassium nitrate concentration. The altered medium (designated Ps minimal) contained 2×10^{-2} M-potassium nitrate instead of the usual 10^{-2} M. Conidiation usually began on the 5th day of incubation. Samples for electron microscopy were collected at different times.

The spray mutant was also incubated at 25° in 50 ml. liquid C-medium or M-minimal medium in 150 ml. Erlenmeyer flasks. These media were similar to Westergaard & Mitchell minimal medium except for a modification in the nitrogen source according to Turian (1964). M-medium contained 10^{-2} M-diammonium citrate; to C-medium 10^{-2} M-dipotassium citrate + 2×10^{-2} M-potassium nitrate were added. Mycelia to be examined were removed on the 3rd day of incubation and immediately placed in fixative.

Light microscopy. Conidia and mycelia were initially observed in the unstained state; however, the use of a cellulose stain was later found to accentuate the visibility of the cell wall. The following procedure was used. Cell material suspended in 0·2 M-hydrochloric acid was placed on a glass slide, stained with a zinc-chlor-iodide reagent (Jensen, 1962), covered with a coverslip, and examined with an O-lux Leitz microscope.

Electron microscopy. Fixation for the preparation of thin sections was done by putting the tissue in 2 %-potassium permanganate for 2 hr at 5°. Post-staining with a saturated solution of uranyl acetate was done, followed by dehydration through a series of graded acetone water solutions. The method of Kellenberger & Ryter (1958) was used for embedding in Vestopal W. Blocks were sectioned with glass knives on a Porter–Blum manual ultramicrotome. Sections were mounted on parlodion-covered grids and all examinations were made with an Hitachi HS-7S electron microscope.

RESULTS

Description of cultures

The spray mutant was not stimulated to produce conidia when cultivated on liquid C-medium; cultures on C-medium and M-medium were identical. In liquid cultures spray formed many unpigmented isolated colonies, presumably the result of growth of the individual conidia of the conidial inoculum. Occasionally after the 8th day of incubation some liquid cultures showed very slight conidiation in the area of the walls of the flask just above the surface of the medium. This conidiation took place in both C-cultures and M-cultures, and only at a few isolated sites along the surface of the medium due to attachment of mycelia there to the walls of the flask. The amount of conidiation was virtually negligible when compared to that of cultures grown on Ps solid medium.

Conidiation and carotenoid production of spray were greatly enhanced on solid Ps medium, where the production of conidia usually began between the 5th and 6th day of incubation. On solid medium the mycelia were characterized by a more restricted growth than wild-type Neurospora. Periodicity was never displayed.

Light-microscope observations

Mycelia were considerably branched and very septate. Conidia showed a wide variety of sizes and shapes. A percentage of spray conidia resembled those found in the wild type; however, the majority were greatly elongated and sometimes showed a reluctance to separate from neighbouring cells (Pl. 1, fig. 1). An interconidial bridge was noted between these attached cells. Conidia were also found which possessed internal conidia (Pl. 1, figs. 2, 3).

Electron microscopy

Except for the cell wall the fine-structure morphology of the spray mutant was similar to that described by Shatkin & Tatum (1959) and Weiss & Turian (1966) for wild-type Neurospora; the cell wall of the spray mutant was, however, considerably thicker and very electron-transparent.

The major difference between the spray mutant and the wild type was in the behaviour on C- and M-media. The width of the mitochondria of spray in C- and M-media was identical with a modal width value of between 0·20 and 0·28 μ, whereas C-cultures of wild type, as reported by Weiss & Turian (1966), had mitochondria which were more swollen than those of M-cultures.

Conidia were grouped in two types by means of electron microscopic examination: ovoid conidia which resembled those found in the wild type (Pl. 2, fig. 6); conidia which served as hosts to internal conidia (Pl. 3, figs. 7, 8). The cell wall of such intraconidial conidia was similar to that of the host cell. The internal conidia contained cytoplasm which had all the normal components, while the cytoplasm of the external cell was always composed of degenerate structures. Finally, an intercellular septum was observed which displayed an internal extension of its wall into the cytoplasm (Pl. 3, fig. 9). This extension may represent an initial stage in the development of internal conidia.

DISCUSSION

The 'spray' mutant behaved differently from wild-type *Neurospora crassa* in C- and M-media. This difference was not only reflected in the gross morphology but also in the fine structure. The lack of swelling of the mitochondria of spray C-medium cultures suggests that the mitochondrial size variations of wild-type *N. crassa* may be related to metabolic processes governing morphogenesis. C- and M-media grown cultures of spray had mitochondria with a modal width between 0·20 and 0·28 μ, a range intermediate to the value obtained by Weiss & Turian (1966) for C- and M-cultures of wild type. It is difficult to relate mitochondrial swelling to metabolic activity; however, the data suggest a connexion between inflated mitochondria and metabolic mechanisms governing conidial differentiation.

An exceptionally thick cell wall was seen in the spray mutant. The composition of cell wall has been implicated in the determination of fungal morphology. Bartnicki-Garcia & Nickerson (1962) reported fine-structure modifications in the thickness of

the cell wall of *Mucor rouxii* and correlated this alteration with quantitative differences in the cell. Mahadevan & Tatum (1965) showed alterations in cell-wall constituents in sorbose-induced and mutated colonial morphological forms of Neurospora. Fine-structure cell-wall variations have been reported for other morphological mutants of Neurospora. Lowry, Durkee & Sussman (1967) pointed out that the 'peach-fluffy' microconidial cell wall was more extensively layered than that of the wild type; Oulevey-Matikian & Turian (1968) found an extremely thick cell wall in the 'amyc' mutant. In view of these findings it is not surprising that the morphological spray mutant displayed an abnormally thick cell wall.

Intra-conidial conidia were observed in the 'spray' mutant. These internal forms had a cell wall which resembled that of the host cell in appearance. All normal cytoplasmic components were found in these internal cells, while those of host cells were always degenerate. This is reminiscent of the intra-hyphal hyphae of the 'clock' mutants described by Lowry & Sussman (1966). Another similarity between 'clock' and 'spray' was the high incidence of moribund hyphae found. However, the 'spray' never displayed periodicity under the growth conditions described.

Kendrick & Molnar (1965), with *Ceratocystis dryocoetidis*, explained the formation of intrahyphal hyphae and endoconidia as the possible result of proliferation of the septum. The median proliferation of the septum that we observed in the spray mutant of *Neurospora crasa* might represent an early stage in the development of intraconidial conidia.

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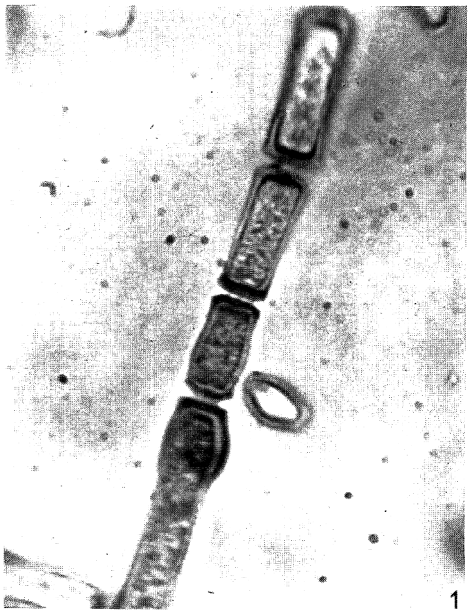

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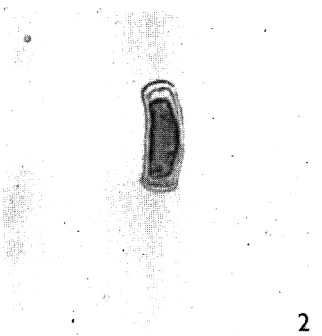

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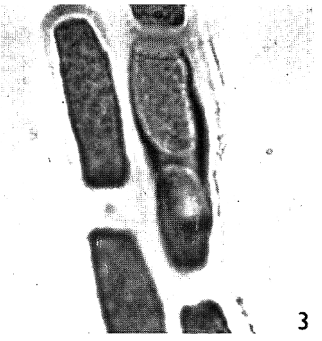

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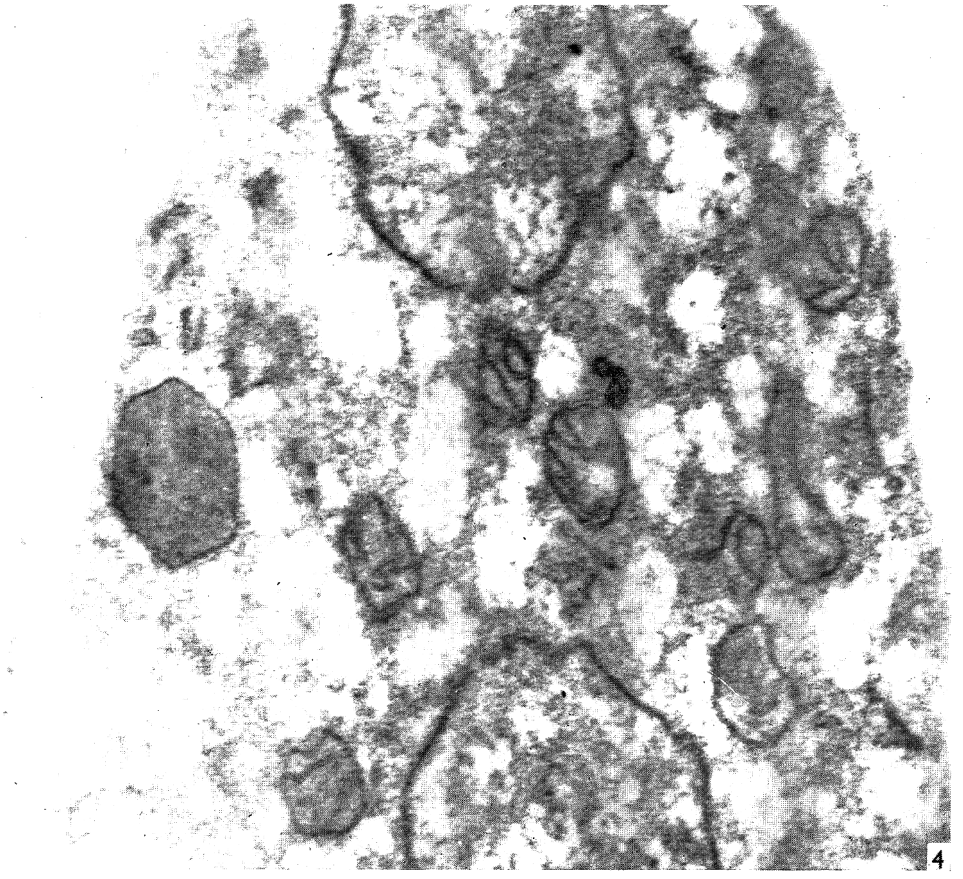

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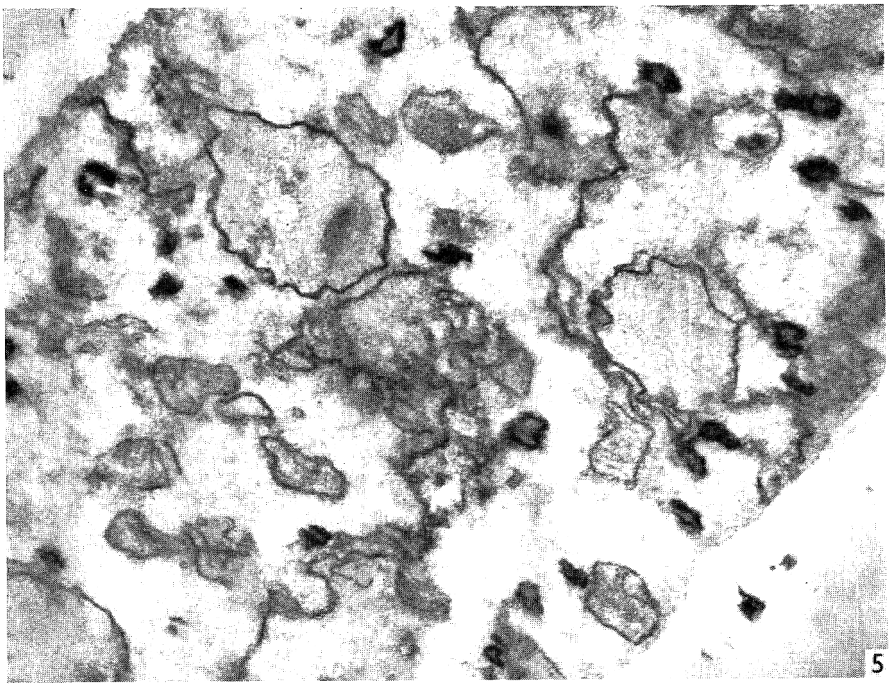

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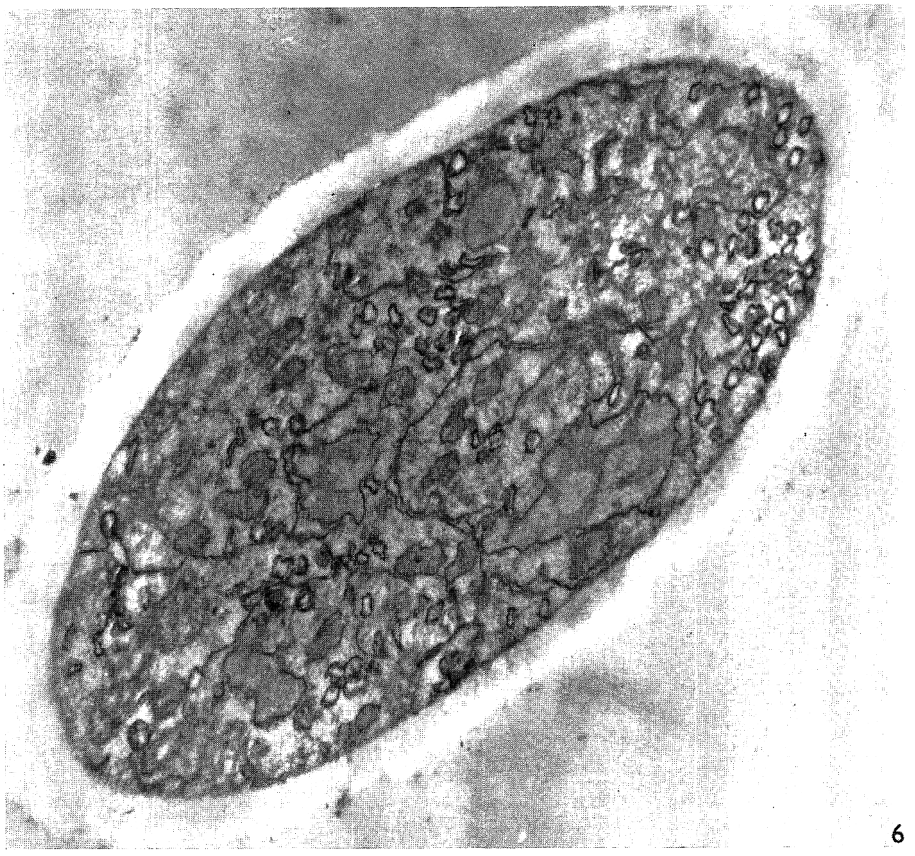

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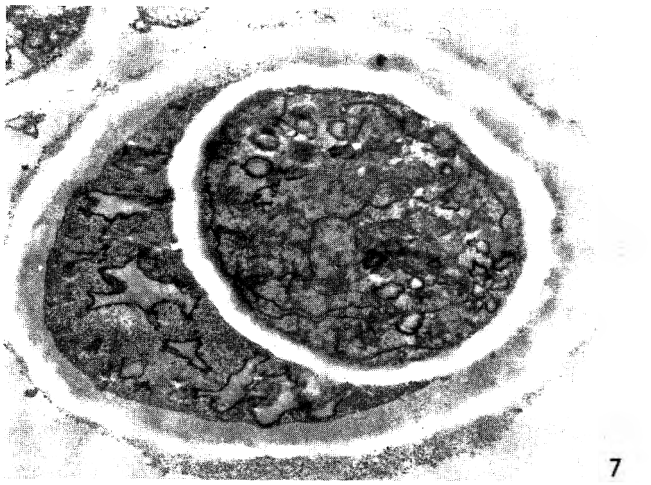

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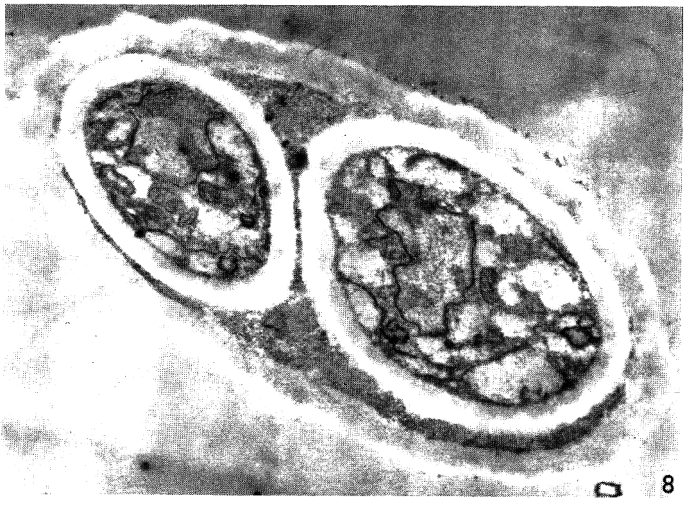

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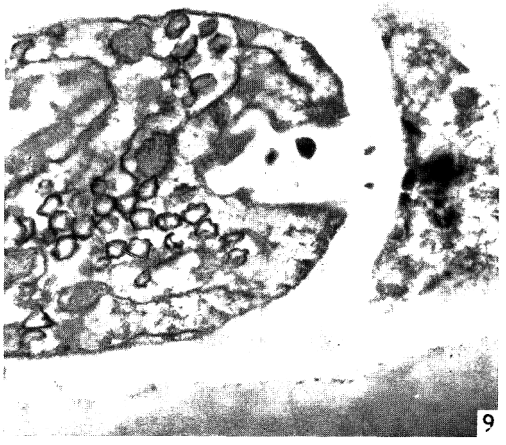

9

EXPLANATION OF PLATES

Light micrographs of *Neurospora crassa* spray mutant cellular material stained with zinc-chlor-iodide reagent; figs. 1–3. All electron micrographs of spray were fixed with $KMnO_4$ and stained with uranyl acetate; figs. 4–9.

PLATE 1

Fig. 1. A chain of elongated conidia interconnected with bridges. Immediately to the right of the chain is an isolated conidium which resembles a normal ovoid wild-type conidium. × 1075.

Fig. 2. Conidium which contains an intraconidial conidium. × 1075.

Fig. 3. The most elongated conidium in this group of conidia can be seen to possess two intraconidial conidia. × 1075.

Fig. 4. A section of hypha taken from a culture grown for 3 days in M-medium. The cytoplasmic components are similar to those grown in C-medium (Pl. 2, fig. 5). On the left is an hexagonal crystal presumed to be ergosterol. × 21,000.

PLATE 2

Fig. 5. A section through a hypha obtained from a 3-day C-medium culture. The cell wall is thick and electron-transparent. The mitochondria have well-defined cristae with restricted matrices, and the endoplasmic reticulum is continuous with the nuclear membrane. × 21,000.

Fig. 6. A section through a conidium obtained from an 8-day culture grown on Ps solid medium. The mitochondria are well defined as are the other cytoplasmic components. The characteristically thick cell wall is present. × 21,000.

PLATE 3

Fig. 7. A conidium containing an intraconidial conidium. Note the moribund cytoplasm of the host cell whereas that of the internal cell is normal. × 21,000.

Fig. 8. A conidium with two internal conidia. Each internal cell possesses a nucleus. Again the cytoplasm of the host cell is moribund. × 21,000.

Fig. 9. Section through a septum showing a median extension of the wall. × 24,500.

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The Location of Mucopolysaccharides on Ultrathin Sections of Bacteria by the Silver Methanamine Staining Technique

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SUMMARY

The silver methanamine technique was used to locate mucopolysaccharides in ultrathin sections of bacteria. Polysaccharides were located along the cell wall and developing cross-walls in young vegetative forms and along the developing cortex in sporulating bacilli. The results are discussed in relation to morphological and chemical studies.

INTRODUCTION

The details of sporulation and germination in bacteria have been extensively studied by the technique of ultrathin sectioning. Species studied include both those in the genus *Bacillus* (Young & Fitz-James, 1959*a*, *b*; 1962; Fitz-James, 1960; Ohye & Murrell, 1962; Kawata, Inoue & Takagi, 1963) and in the genus *Clostridium* (Hashimoto & Naylor, 1958; Takagi, Kawata & Yamamoto, 1960; Fitz-James, 1962). More recently electron-microscope studies have been combined with immunological and cytochemical studies to demonstrate the location of antigens (Thomson, Walker & Hardy, 1966; Walker, Baillie, Thomson & Batty, 1966; Walker, Thomson & Baillie, 1967*a*, *b*) and enzymes (van Iterson & Leene, 1964*a*, *b*; Sedar & Burde, 1965*a*, *b*; Baillie, Thomson, Batty & Walker, 1967) during various phases of growth.

Proteins and nucleic acids have been located on ultrathin sections of bacteria by observing the changes in structure produced following extraction of these components by digestion with specific enzymes (Granboulan & Leduc, 1967). Although proteins could be digested by proteinases in ultrathin sections of osmium-treated cells (Monneron & Bernhard, 1966), satisfactory digestion of nucleic acids with nucleases was only obtained after aldehyde fixation and the use of a water-soluble resin (Granboulan & Leduc, 1967). Nermut (1967) attempted to stain ultrathin sections of *Bacillus megaterium* for polysaccharides using Ruthenium red, although the results were not very satisfactory. Various methods have been devised for staining ultrathin sections of animal tissues to demonstrate carbohydrates such as glycogen, mucopolysaccharides and glycoproteins. One of these, the silver methanamine technique, was first devised for conventional light microscopy (Gomori, 1946; Jones, 1957) but was later extended to the demonstration of polysaccharide-rich structures in tissues using the electron microscope (Churg, Mautner & Grisham, 1958; De Martino & Zamboni, 1967). In the present paper modifications of this technique are described for the staining of bacterial polysaccharides during phases of sporulation and germination.

METHODS

Organisms. The following organisms were used: *Clostridium bifermentans* (Wellcome Research Laboratories collection) CN1617; *C. botulinum* type C CN4946; *C. sporogenes* (Leeds University Bacteriology Department collection) L206; *Bacillus cereus* var. *terminalis*.

Sporulation. In the case of *Clostridium bifermentans* and *C. sporogenes* the deposit from 25 ml. of a 6 hr sporulating culture in heart infusion broth (Difco) was fixed for 18 hr in the fixative of Kellenberger, Ryter & Séchaud (1958). Similar deposits of sporulating bacteria of *C. botulinum* type C and *Bacillus cereus* were prepared from 18 hr cultures in Robertson meat broth and the sporulating medium of Young (1958), respectively.

Germination. Spore suspensions of *Clostridium bifermentans* and *Bacillus cereus* were heat-shocked at 65° for 10 min. and incubated in nutrient broth containing 5 mM-L-alanine. Samples were removed after 5 min. and 1 hr for centrifugation and fixation.

Preparation of ultrathin sections. After fixation all specimens were dehydrated in ethanol and embedded in Maraglas (Freeman & Spurlock, 1962). Sections were cut with an L.K.B. 'Ultratome' on to distilled water and transferred by means of a wire loop on to the various staining solutions. After the final rinse the sections were collected on 200-mesh formvar-coated grids and examined in a Philips EM 200 electron microscope at 60 kV.

Staining solutions and method of staining. The method used was essentially that of Marinozzi (1961) as modified by Short (1968). To 10 ml. 0·25 % (w/v) silver nitrate add 10 ml. absolute ethanol, 0·3 g. methanamine and 10 ml. distilled water.

Method. Float the sections on to 0·5 % periodic acid, to oxidize them, for 10–20 min.; wash in two changes of distilled water; transfer to the silver solution in a covered receptacle and leave for 2 hr at room temperature (about 22°); wash thoroughly four or five times in distilled water; transfer to 0·5 % sodium thiosulphate for 1–2 min.; wash in distilled water; mount on grids.

RESULTS

The results of staining ultrathin sections of *Clostridium bifermentans* with silver are shown in Pls. 1, 2 and 3; in each case similar sections are compared before and after treatment. It can be seen in Pl. 1, fig. 1 that silver deposits were arranged along the cell wall of the young vegetative form and along the developing cross-wall, with little staining of the cytoplasm; there was considerable difference in contrast between the oxidized and unoxidized sections. Staining of the developing spore is shown in Pl. 2, fig. 2. Both the vegetative cell wall and the developing cortex are stained with silver grains. In the section of germinating spores, shown in Pl. 3, fig. 3, only the cell wall of the emerging vegetative form is stained.

A similar picture is shown for sporulating forms of *Clostridium botulinum* type C (Pl. 4, figs. 4, 5) and *C. sporogenes* (Pl. 4, fig. 6). In both cases the cell wall and developing cortex, but not the spore coat, were stained by silver deposits. Very similar results were found both in sporulating forms and in germinating spores of *Bacillus cereus*. In freshly germinated spores of *B. cereus* only the region of the disintegrating cortex and developing vegetative cell wall was stained (Pl. 4, fig. 7).

DISCUSSION

Staining of carbohydrates results from the reduction of the silver solution by free aldehyde groups released from the mucopolysaccharides by oxidation; electron-dense silver deposits are thus formed at the site. Oxidation also serves to re-oxidize reduced osmic acid which would, if not removed, also reduce silver. During fixation of biological specimens with osmic acid, lower oxides of osmium are formed which blacken the cells and are responsible for much of the electron-density of the sections. Treatment of sections with periodic acid re-oxidizes the reduced osmium by converting it to the soluble tetroxides. This results in a decrease in electron-density in the specimen, particularly in the membranes (Silva, 1967).

The development of silver grains along the cell wall is in agreement with the demonstration of polysaccharide components in isolated walls of both Gram-positive and Gram-negative bacteria by Salton (1953). Staining of the cortex is also explicable by reference to its mode of development and to chemical studies on disintegrated spores. It is known that the cortex is secreted between the two membranes of the fore-spore, which develops as an invagination of the cytoplasmic membrane (Fitz-James, 1960; Ohye & Murrell, 1962). The cortex is in close contact with the 'outer' side of the cytoplasmic membrane, which would normally be in contact with the bacterial cell wall and can thus be regarded, in part, as an endogenous cell wall (Warth, Ohye & Murrell, 1963*a*, *b*). From their chemical studies on disintegrated spores these workers have also suggested that the spore cortex and bacterial cell wall have a similar chemical composition. Their observation that the spore coat and exosporium consist mainly of protein is supported by our observation that membranes of neither were stained with silver grains.

In some previously reported studies the chemical analyses of various fractions of bacteria have been complicated by the fact that these have not always been homogeneous. For example, it was pointed out by Warth *et al.* (1963*b*) that while the analysis of spore-coat preparations by several workers had indicated the presence of small amounts of hexosamine or DPA material, this could be explained by the presence of small amounts of adherent cortical material in their preparations. The advantage of direct staining is that the chemical components can be identified *in situ* without any undue distortion of the structure. As far as is known this is the first successful application of this method to the demonstration of bacterial carbohydrate *in situ*.

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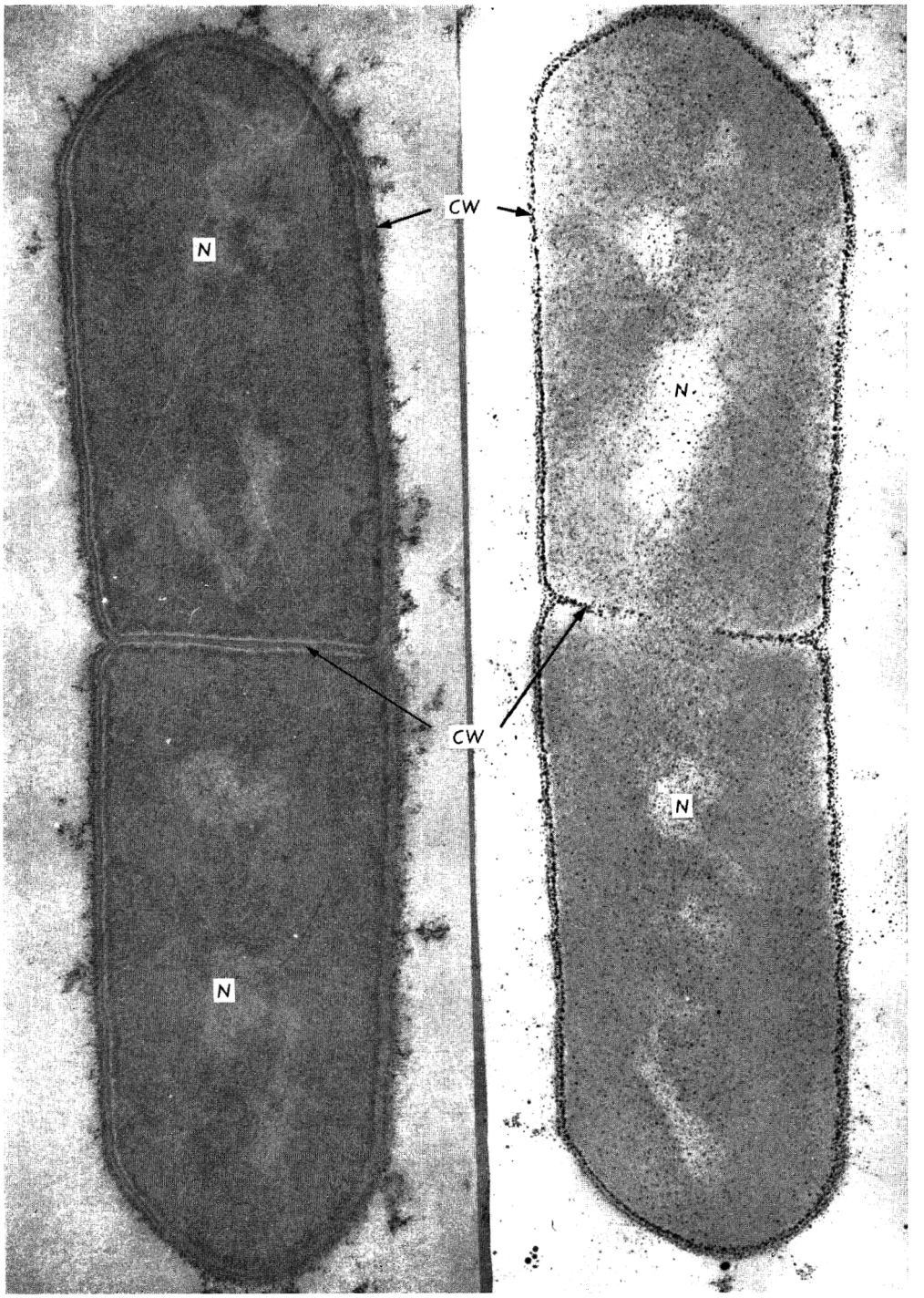

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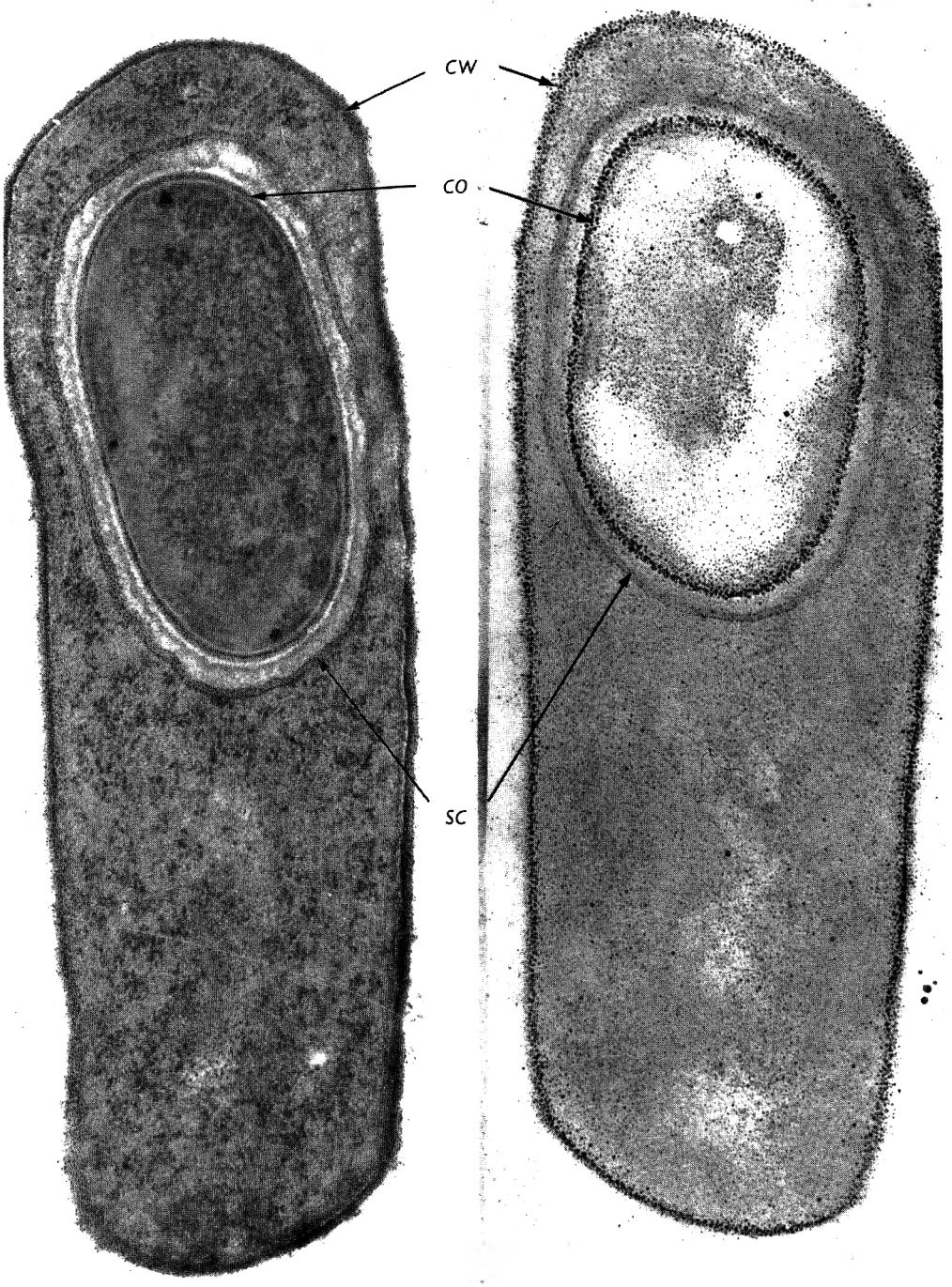

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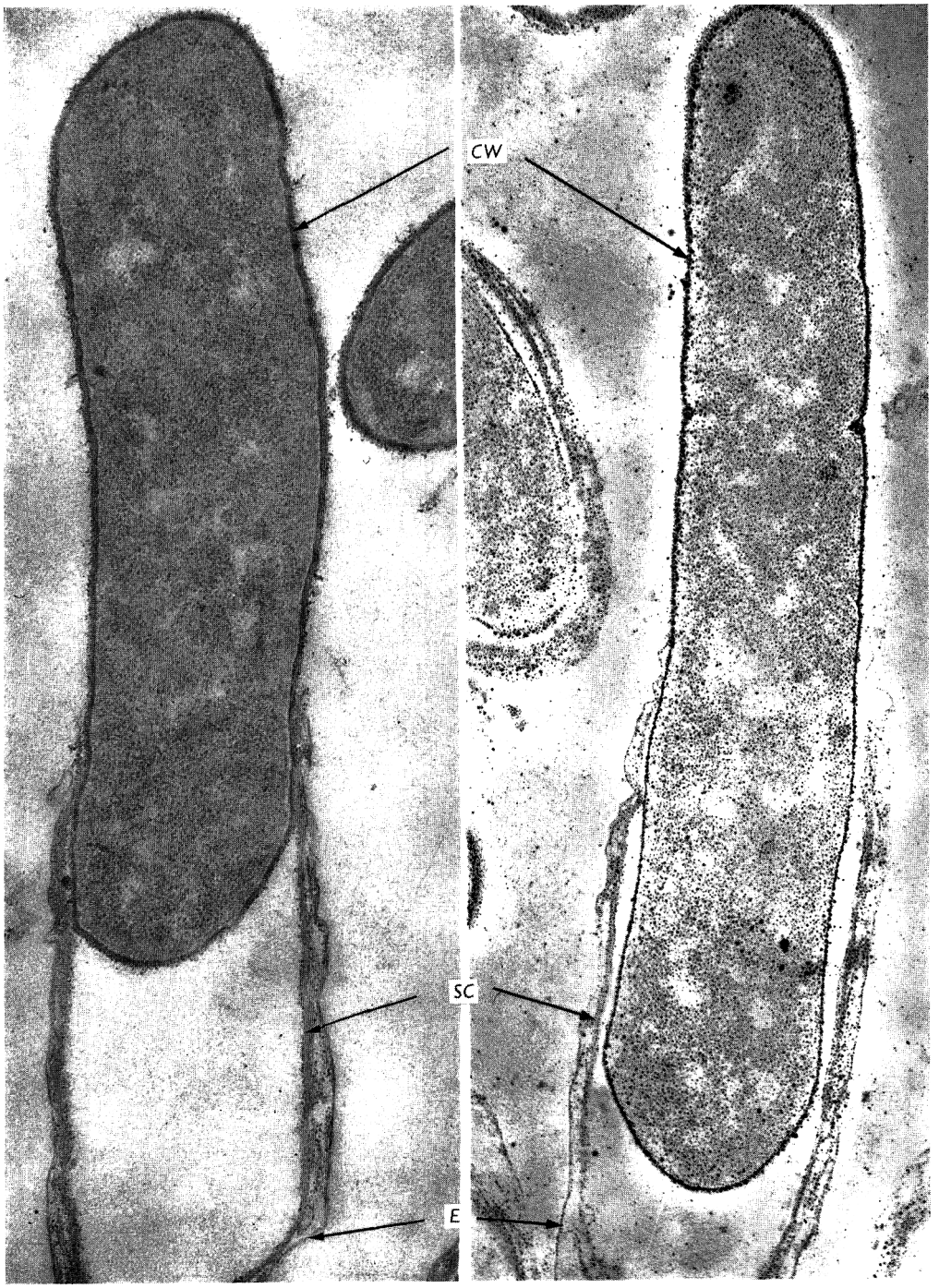

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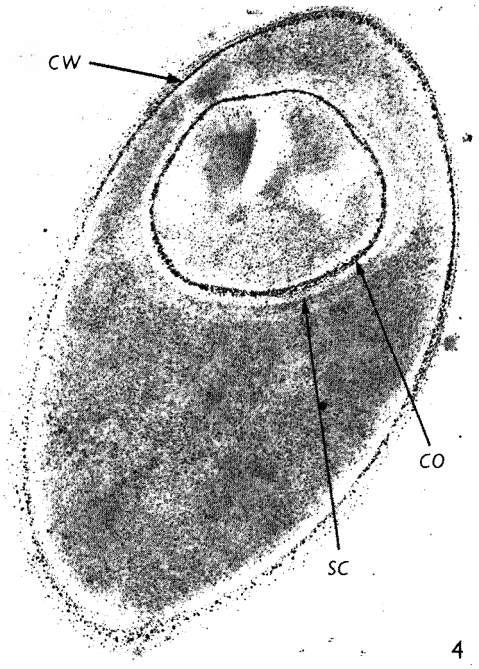

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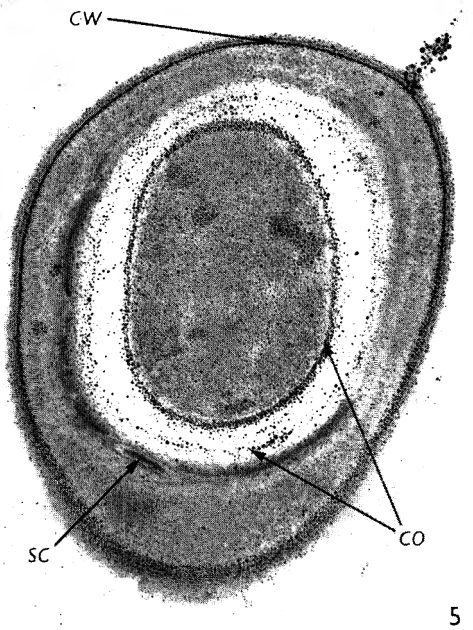

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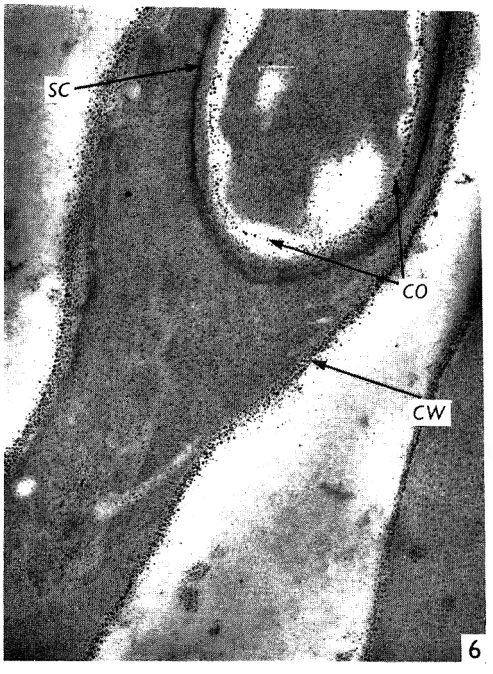

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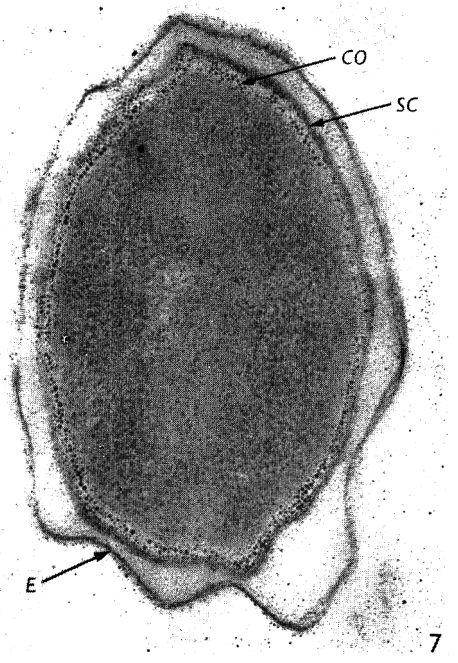

7

EXPLANATION OF PLATES

Abbreviations used: *CW* = cell wall; *CO* = cortex; *SC* = spore coat; *E* = exosporium; *N* = nuclear material.

PLATE 1

Fig. 1. Section of young vegetative cells of *Clostridium bifermentans* CN1617 unoxidized (L) and oxidized with periodic acid (R); stained with silver. Silver grains can be seen along the cell wall and developing cross-wall. × 65,000.

PLATE 2

Fig. 2. Section of sporulating cells of *C. bifermentans* CN1617 unoxidized (L) and oxidized with periodic acid (R); stained with silver. Deposits of silver are located along the cell wall and cortex, not the spore coat. L, × 60,000; R, × 76,250.

PLATE 3

Fig. 3. Section of germinating spore of *C. bifermentans* CN1617, showing outgrowth after 1 hr incubation unoxidized, L; and oxidized with periodic acid, R; stained with silver. Deposits of silver grains are seen along the cell wall of developing vegetative cell, but not along the spore coat or exosporium. L, × 54,400; R, × 46,000.

PLATE 4

Figs. 4, 5. Section of sporulating cells of *C. botulinum* type C CN4946 after oxidation and staining with silver. Deposits of silver are arranged along the vegetative cell wall and along the cortex. Fig. 4, × 45,750; fig. 5, × 61,000.

Fig. 6. Section of sporulating cells of *C. sporogenes* L206 after oxidation and staining with silver. Deposits of silver are evident along the vegetative cell wall and cortex, but not the spore coat. × 45,750.

Fig. 7. Section of germinating spore of *Bacillus cereus* var. *terminalis* (5 min. incubation), after oxidation and staining with silver. Deposits of silver are evident along cortex and developing vegetative cell wall. × 93,500.

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