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THE JOURNAL OF GENERAL MICROBIOLOGY

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

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

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Descriptions of new species of cultivable microbes should not be submitted unless an authentic specimen of a living culture has been deposited in a recognized culture collection.

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Except for good reasons, micro-organisms should be designated by the names used in the works listed below. When other authorities are followed, they should be cited whenever obscurity might result from their use.

MICROFUNGI. *Ainsworth & Bisby's Dictionary of the Fungi*, 1961, 5th ed. (Kew: Commonwealth Mycological Institute.)

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

PLANT VIRUSES AND VIRUS DISEASES (1957). *Rev. appl. Mycol.* **35**, Suppl. 1–78.

BACTERIA. Author's references in naming are at present accepted provided that the designation is unambiguous and conforms with the International Bacteriological Code of Nomenclature (1949; *J. gen. Microbiol.* **3**, 444) and the Opinions issued by the International Committee on Bacteriological Nomenclature. If desired, a synonym may be added in brackets when a name is first mentioned.

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Micro-Organisms in the Intestines of Earthworms

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SUMMARY

Actinomycetes and bacteria, but not fungi, increase rapidly in numbers during the passage of food through the worm gut. Enzymes produced by the worm rather than micro-organisms seem to be the main agents digesting cellulose and chitin. The rate that material moves through the intestine depends on whether the animal is feeding; food takes about 20 hr. to pass, but when burrows are being formed material passes in about 12 hr.

INTRODUCTION

The influence of earthworms on soil formation and in changing the structure of agricultural soil has received considerable attention, but little has been published on the effects of worms on the soil microflora. Bassalik (1913) found no qualitative difference between the microflora of the worm gut and surrounding soil, but Aichberger (1914) found the worm gut to differ from the soil in containing no lice, yeasts, desmids, rhizopods, or blue green algae. Dawson (1947) compared aggregates formed in the worm intestine with those from casts and soil and found fewer bacteria in intestinal aggregates but no differences in the fungi. By using an enrichment technique Khambata & Bhat (1953, 1955, 1957) studied specific groups of bacteria in the worm intestines and isolated oxalate and cellulose decomposers, but no nitrifying organisms. Hutchinson & Kamel (1956) isolated viable thick-walled and thin-walled fungal spores from worm gut and showed that the fungi were dispersed more rapidly in sterilized soil containing worms, than when worms were absent. The present work follows the changes in the microbial population in ingested material as it passes through the worm, and attempts to evaluate benefits the worm may derive therefrom.

METHODS

Earthworms. Three species were studied: *Lumbricus terrestris* (Linn.), *Allolobophora caliginosa* (Sav.) and *Allolobophora terrestris* (Sav.) form *longa* (Ude), which will be referred to as *A. longa* to avoid confusion with *L. terrestris*. All specimens were collected from the same soil at Rothamsted, a flinty silt loam pH 6·5 of the Batcombe Series, which had been under permanent pasture for 70 years (Great Field IV).

Media. Soil extract agar (Lochhead & Burton, 1956) was used to count bacteria, and sodium asparaginate agar (Conn, 1921) for actinomycetes. Filamentous fungi

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were counted on a glucose peptone agar, containing 10% glucose, 0·5% peptone, 0·1% KH_2PO_4, 0·05% $MgSO_4,7H_2O$ and 2% agar, and yeasts on Sabouraud agar (Anderson, 1917) both of which were at pH 7·0 and contained 30 mg. aureomycin/ml., added just before inoculation to inhibit growth of bacteria. For chitinolytic organisms a medium devised by Veldcamp (1955) was used and for cellulose decomposers Jensen's (1940) mineral salt solution with filter-paper strips. All media, except Sabouraud agar, were dispensed in 4 ml. lots in test tubes. The yeasts when grown in tubes were obscured by other fungi and were therefore surface inoculated on to Petri plates.

Cellulose suspension for metabolic studies was prepared from high-quality cotton-wool broken up by soaking overnight in conc. HCl at 5°. This was then dispersed in a large volume of water and further macerated in a Waring blender. The fine particles were separated by decantation, ground for 24 hr. in a ball mill, and the slurry formed was diluted with water to give a suspension containing 2% (w/v) of cellulose.

Chitin suspension was prepared from the shells of *Sepia officinalis* by the method of Clarke & Tracey (1956).

Reducing sugar was determined by the modified Somogyi–Nelson method (Nelson, 1944) after precipitation of protein with $ZnSO_4/Ba(OH)_2$ reagents. Acetyl glucosamine was determined by the method of Morgan & Elson (1934).

Collection of samples and counting procedure. Earthworms were collected by digging, and at the same time soil samples of 40–60 g. were taken. Worms were washed free from soil and batches of five were killed by brief immersion in water at 48° and dissected immediately. Samples of gut contents of about 0·5 g. dry weight were placed in 50 ml. sterile distilled water in weighed screw-capped bottles, which were shaken at 200 strokes per min. for 20 min. A series of two-fold dilutions was prepared from this suspension by using silicone-coated pipettes which were changed frequently. Two samples were taken from each dilution into each culture medium. Tube cultures were sloped to give a thin layer of medium to facilitate counting. The number of organisms present was estimated from the total number of cultures showing growth (Fisher & Yates, 1938).

Soil samples were sieved through 1 mm. mesh and subsamples were taken for a similar dilution series. When dilutions had been prepared, the original samples were dried at 105°, weighed and all counts corrected to numbers per g. dry weight of soil or gut contents.

RESULTS

The microflora of the gut contents of three species of worm

Table 1 gives counts on composite samples from all sections of the gut of *Lumbricus terrestris*, *Allolobophora caliginosa*, and *A. longa*. For the first two species, which feed actively for most of the year, the samples were collected during June and July. Over this period *Allolobophora longa* was aestivating, with an almost empty gut containing many fewer organisms than soil. The *A. longa* counts were from feeding worms collected in September and October.

Many more actinomycetes and bacteria were isolated from the gut of each species than from the soil. The differences between the soil and gut counts were highly significant, in spite of the large variation between samples. *Allolobophora caliginosa*

contained more yeasts than *Lumbricus terrestris* probably because it was eating more; these increases over the numbers isolated from the soil were not significant. Sample variances of fungal counts of both soil and gut contents were large. The

Table 1. *Counts of yeasts, fungi, actinomycetes and bacteria in worm intestinal contents compared with soil. Mean log. number of organisms per g. dry wt. of gut contents or soil*

	Yeasts	Fungi $\times 10^6$	Actino-mycetes $\times 10^6$	Bacteria $\times 10^6$
		Lumbricus terrestris		
Gut content	4·39	−0·25	1·97	4·91
	±0·122	±0·122	±0·154	±0·166
Soil	4·70	−0·50	0·95	2·07
	±0·178	±0·164	±0·226	±0·244
		Allolobophora caliginosa		
Gut content	5·06	−0·08	3·12	4·42
	±0·070	±0·081	±0·198	±0·342
Soil	4·79	−0·39	1·54	2·06
	±0·103	±0·119	±0·290	±0·500
		A. longa		
Gut content	5·46	0·52	3·25	5·30
	±0·074	±0·068	±0·102	±0·070
Soil	5·00	0·15	1·00	2·03
	±0·109	±0·099	±0·149	±0·103

Fig. 1. Number of bacteria and actinomycetes in the various sections of the gut of *L. terrestris*. ■, bacteria; □, actinomycetes.

differences between the number of fungi recovered from different samples probably depended on differences in the fungal content of the food. When counts were very high, one species, usually a Penicillium, was dominant.

Both actinomycetes and bacteria increased greatly in the gut of *Allolobophora longa* as with the other species, but this worm also contained more fungi and yeasts

than soil (significant at the 5% level). The reasons for these small increases are not clear; they may have come from differences between the soil sampled and the actual material eaten by the worm.

Actinomycetes and bacteria in different sections of the gut were counted in samples of 25 *Lumbricus terrestris*, collected in groups of five. After dissection, the intestine was divided into the following sections, each of which was measured; fore-gut (buccal cavity, oesophagus, crop and gizzard), mid-gut and hind-gut, and a separate sample was taken from each. The results in Fig. 1 are plotted along an abscissa representing the length of the worm's intestine with the counts entered in positions corresponding to the middle of each section of gut. The bacteria and actinomycetes increased in number throughout their passage through the worm, the increases tending to be logarithmic.

Cellulolytic activity in the intestine of earthworms

Worms feed largely on dead plant and insect material and the large increase in the bacterial and actinomycete populations during passage of food through the gut suggests that cellulolytic (and chitinolytic) organisms might be selectively increased. Tracey (1951) found evidence for the presence of cellulase and chitinase in aqueous extracts of several earthworm species but did not determine the source of these enzymes.

Counts of cellulose decomposers in the intestinal contents were irregular and an indirect method based on oxygen uptake (Schmidt & Ruschmeyer, 1958) was used. Samples of gut contents of *Lumbricus terrestris* of approximately 1 g. dry weight of material were suspended in 10 ml. phosphate buffer (pH 7·0). Warburg flasks were prepared containing 3 ml. of this suspension and with 0·5 ml. 2% cellulose suspension as substrate; in control flasks 0·5 ml. water replaced the cellulose substrate. Flasks were shaken and oxygen uptake was measured at 25°. Readings were made hourly for 12 hr. on 3 consecutive days (Chase & Gray, 1957); the results are shown in Table 2.

Table 2. *Oxygen uptake of gut contents from* Lumbricus terrestris

	μl. oxygen/hr.	
Day	Control	+ Cellulose
1	12·8	12·9
2	16·2	17·8
3	22·0	29·8

Initially the O_2 uptake was the same for all flasks, but after a short period the respiration rate in those with cellulose increased greatly. This might reflect either the presence of cellulolytic organisms, or the production of glucose by a cellulase from the worm and its metabolism by micro-organisms. Fewer than 1000 cellulolytic organisms per g. dry weight were recovered from flask contents at the end of the 3-day period, so experiments were made to test for a cellulase.

Cellulolytic activity of total gut contents

Gut contents were suspended in buffer as described and duplicate sets of flasks prepared with and without added cellulose. To each was added 0·5 ml. toluene to stop bacterial activity. Flasks were incubated at 25° and samples taken at 24 hr. intervals were tested for glucose (Table 3). The experiment was repeated with gut contents from mid- and hind-gut sections (Table 4).

Table 3. *Glucose production of total gut contents of* Lumbricus terrestris

	μg. glucose/ml.	
Day	Control	+Cellulose
0	209	209
1	232	246
2	236	256
3	244	264

Table 4. *Glucose production from mid- and hind-gut contents of* Lumbricus terrestris

	μg. glucose/ml.	
Day	Control	+Cellulose
Mid-gut contents		
0	216	216
1	198	252
2	243	378
3	342	729
Hind-gut contents		
0	160	160
1	144	160
2	216	208
3	224	208

The results show that an appreciable amount of glucose was present in the freshly prepared suspensions. This increased during incubation, particularly with added cellulose, indicating the presence of cellulolytic enzymes in the absence of bacterial activity. Table 4 shows a higher cellulase activity in the mid-gut section.

Further work on the source of the cellulase was done using gut wall, washed free from the contents. Finely ground portions of the gut wall were suspended in buffer with cellulose and toluene and incubated at 25°. Table 5 shows the results per ml. of suspension containing 0·5 g. wet weight of gut wall.

These results confirm those in Table 4 in showing that cellulose decomposition is restricted to the mid-gut and provide strong evidence that the enzyme is secreted by the worm rather than by organisms occurring in the intestine. The appreciable amounts of glucose in the control series came from the gut wall, and was highest where cellulose decomposition was most active.

Glucose production in toluene-treated suspensions of washed intestinal contents was correlated with oxygen uptake in the Warburg apparatus. Table 6 shows that

the uptake of oxygen was small and unaffected by the addition of cellulose, indicating that washing removed the enzyme.

Microscopic examination of the intestinal contents stained with chlor-zinc-iodine and of unstained material with polarized light gave no evidence of breakdown of cellulose by bacteria.

Table 5. *Glucose production from cellulose by gut wall suspensions of* Lumbricus terrestris

Day	μg. glucose/ml. Control	+ Cellulose
	Fore-gut wall	
0	160	160
1	360	400
2	480	480
3	490	480
	Mid-gut wall	
0	595	595
1	854	883
2	960	1210
3	1037	1378
	Hind-gut wall	
0	240	240
1	444	384
2	612	576
3	792	614

Table 6. *Glucose production and oxygen uptake by washed gut contents of* Lumbricus terrestris

Day	μg. glucose/ml. Control	+ Cellulose
0	30	30
1	36	37
2	45	44
3	54	52
	Average oxygen uptake μl./hr.	
0	10·8	12·1
1	11·1	11·4
2	16·2	16·0
3	14·0	14·4

Chitinase activity in earthworms

Microscopic examination of the intestinal contents showed numerous fragments of chitinous material, mainly insect cuticle. Many bacteria attack chitin (Veldkamp, 1955; Clarke & Tracey, 1956): this complicates the problem of deciding whether it is the earthworm or the micro-organisms which produce the chitinase found by Tracey (1951). Chitinase activity and the number of chitin decomposers were measured in different parts of the gut content and gut wall. In principle these

experiments were similar to those on cellulose breakdown. Acetylglucosamine production was measured after incubation of gut contents with chitin. Tables 7 and 8 show chitinase activity in gut content and gut wall. Acetylglucosamine production by gut content was small, near the limit detectable by the method, but was always larger with chitin. The high values obtained from the fore-gut wall (Table 8) indicated the presence of chitinous material other than that added in the experiment. This was probably the horny material from the gizzard, and in a second experiment this was removed by dissection as far as possible. This was only partly successful; similar results were obtained, the mid- and hind-gut having higher glucosamine contents in the presence of chitin (Table 9).

Table 7. *Chitinase in gut content of* Lumbricus terrestris

Day	μg. acetyl glucosamine/ml. Control	Chitin
	Mid-gut	
0	1·5	1·5
1	2·0	3·5
2	1·5	3·5
	Hind-gut	
0	0·6	0·8
1	0·8	4·5
2	1·4	5·8

Table 8. *Chitinase in gut wall of* Lumbricus terrestris

Day	μg. acetyl glucosamine/ml. Control	Chitin
	Fore-gut	
0	3·5	3·5
1	12·0	11·0
2	20·0	15·5
	Mid-gut	
0	2·2	2·2
1	2·1	3·3
2	3·0	5·1
	Hind-gut	
0	1·8	1·8
1	2·1	4·5
2	3·0	3·6*

* This sample became contaminated and acetylglucosamine was destroyed by bacteria.

Table 10 shows that, on average, chitin-decomposing micro-organisms were ten times more abundant in the hind-gut than in the mid-gut. These results suggest that chitin breakdown is promoted by bacterial activity as well as by enzymes produced by the worm.

Rate of food movement through the worm intestine

In the results reported so far, attention was paid only to factors affecting the food material as it passed through the earthworm intestine. Few conclusions can be drawn about the effect of worms on either the microbial population or the ingested material, unless the time required for food to pass through the worm is known.

Table 9. *Chitinase in gut wall of* Lumbricus terrestris

Day	µg. acetyl-glucosamine/ml. Control	Chitin
	Fore-gut	
0	12·5	12·5
1	37·5	37·5
2	50·0	50·0
3	56·2	56·2
	Mid-gut	
0	13·3	13·3
1	16·6	23·3
2	20·0	33·3
3	23·3	36·7
	Hind-gut	
0	11·1	11·1
1	13·9	25·0
2	13·9	33·3
3	16·7	41·7

Table 10. *Chitin decomposers/g. dry wt. in gut contents of* Lumbricus terrestris

Sample	Number in millions/g. dry wt. Mid-gut	Hind-gut
1	17·9	228·8
2	37·3	114·4
3	38·2	786·2
4	28·6	112·4
5	53·1	632·3
Mean	35·0	374·8

Something is known about food movement in insects. Snipes & Tauber (1937) studied *Periplaneta americana*, Wedberg & Clarke (1947) *Blaberus cranifer*, and Sinha (1958) the stored-grain beetle, but these insects were starved for a period before the experimental feeding. This was considered undesirable for the present investigation because nothing appears to be known about the effects of starvation on food movement.

Several compounds were tried as markers of either food or soil. Sudan IV was not readily recovered and Malachite green poisoned worms. Diatomaceous earth was also tried, but the 5% which it was necessary to add so irritated *Allolobophora*

caliginosa that the material was passed through the gut in 2 hr. These markers had the further disadvantage that the worms had to be killed to see how far the material had travelled. In an attempt to avoid these difficulties ^{32}P was used to mark soil.

Two lots of 100 g. dry soil were weighed into bottles and ^{32}P as H_3PO_4 added in 25 ml. water to give 10 μc./100 g. and 50 μc./100 g. Soils were allowed to equilibrate overnight and the next day five large *Lumbricus terrestris* were placed in each. Three hours later the worms were removed, adhering soil washed off and a Geiger counter used to measure the distance travelled by the marked soil. Dissection showed that with worms receiving 50 μc./100 g. an end-point could be measured accurately on live worms. This method was applied to worms removed at intervals from soil, with the results shown in Fig. 2. The distance moved relative to worm-

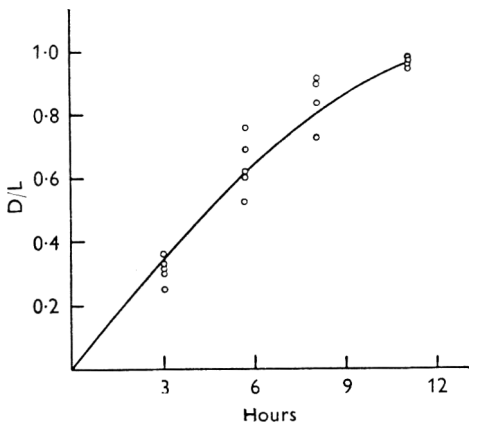

Fig. 2

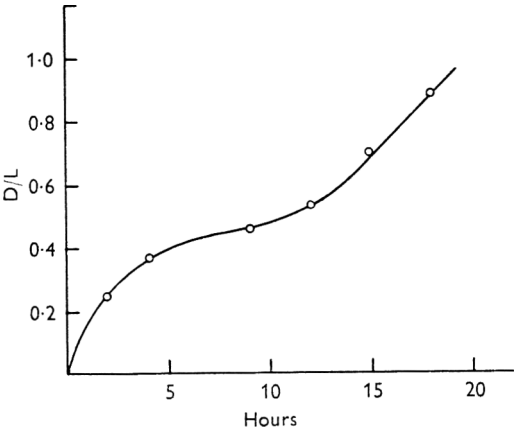

Fig. 3

Fig. 2. Movement of soil through gut of *L. terrestris*.

Fig. 3. Movement of food through gut of *L. terrestris*.

length is plotted against time to allow for variation in the size of worms. This experiment gave a value of 11–12 hr. for the full passage of soil and food through the gut of *L. terrestris*, which is too short a time for the soil bacteria to multiply to the extent found in earlier experiments. An explanation for this result was found in the experimental method. Each time the worms were returned to the soil they were forced to make fresh burrows by ingesting soil and so were not, in fact, feeding. To overcome this objection the food instead of the soil was marked. Of several materials tried small pellets made from dung mixed with a dark peat soil were most satisfactory. Mature *L. terrestris* were kept singly for 48 hr. in pots of Barnfield soil, which contains little organic matter, to allow them to make burrows. Three pellets were placed on the surface of the soil in each pot, the pots were then examined at hourly intervals, and the disappearance of pellets recorded. At intervals after the pellets had been taken worms were removed, killed and dissected and the position of the labelled dung+peat recorded. Figure 3 shows the results. Each point represents only one worm; replication was impractical because the worms fed sporadically; on some nights no food was taken. Accuracy was also limited by the fact that observations were made only hourly; nevertheless, it could be concluded that food probably remains in the gut of *Lumbricus terrestris* for about

20 hr. A similar value was obtained by Barley (1959) for *Allolobophora caliginosa*. He also suggested that soil travels more rapidly through the gut of worms when they are burrowing. The flat portion of the above graph where the food travelled more slowly corresponds to the mid-gut, the region of greatest enzyme activity.

DISCUSSION

A major difficulty in comparing soil itself and soil in the gut of a soil-eating animal is in obtaining comparable samples. Earthworms when feeding not only ingest soil and plant material in different proportions at different times, but they also take in soil non-selectively when making fresh burrows. Pot experiments offer a measure of control impossible in the field, but may introduce other sources of error. An attempt was made to overcome this uncertainty by using a large composite sample from the top few centimetres of soil which includes much of the organic matter in the surface layer upon which worms normally feed. Although this is not an ideal solution, because an empirical choice is involved, the counts obtained for yeasts and filamentous fungi, which did not reproduce in the gut, were substantially the same for soil and gut contents, indicating that the worms ingested material similar to that taken as soil samples. The associated problems of sampling variance were overcome by taking large primary samples and subsampling as required.

Population estimates by the dilution tube technique were much greater than any reported using the plate count. At the highest dilutions showing growth in this method only one or two organisms occurred per culture, so that interference between colonies, well known to be one of the causes of anomalous plate counts, was decreased. Another factor was the use of soil extract agar which gives higher counts than more selective media.

Actinomycetes and bacteria in the gut flora increased greatly in number in all three species of worms. Conditions were less suitable for yeasts or filamentous fungi, which usually did not increase in the gut. The results suggest that changes in the microbial population during passage through the worm gut tended to be logarithmic, indicating that increases were by bacterial growth and not by the worm selecting food material with a high bacterial count. These results are contrary to those of Day (1950) who examined worms kept in pots, where they may not have been feeding normally.

There seems little doubt that cellulose is broken down in the earthworm intestine largely by enzyme secreted by the animal and not by micro-organisms present in the gut. The length of time the food remains in the intestine would not allow much microbial breakdown unless there were very many cellulolytic organisms, and counting did not show a large population of these.

The mode of breakdown of chitin is less clear, but the worm seems to produce some chitinase and there are many organisms in the gut which are able to attack it. How much chitin and cellulose are broken down during passage through the earthworm is not known, but cast material contains considerable amounts of these materials. The low temperature and short residence of food in the gut would not allow the breakdown of much resistant material. No evidence was obtained to suggest that the worm had a specialized gut flora, quantitatively different from that of the soil in which it was feeding. Neither could it be shown, with the possible exception of chitin-decomposing bacteria, that the organisms present helped the worm to digest its food.

The reason for the increase in numbers of bacteria and actinomycetes remains unexplained. In the absence of evidence that they take a major part in cellulose and chitin breakdown, their increase may come partly from sugars etc. released by enzymic degradation of organic material, and partly from the mechanical action of the worm's oesophagus and gizzard in breaking down the organic matter into a finely divided state in which it is more readily susceptible to microbial attack.

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A Microbiological Study of Earthworm Casts

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SUMMARY

Microbiological, physical and chemical changes were followed in worm casts ageing in the field. Filamentous fungi and yeasts increased in number rapidly after the cast was produced, but not bacteria or actinomycetes which were initially numerous. Measurements of hyphal length confirmed the increased growth of fungi. Ageing casts showed a declining respiratory activity, possibly because the bacteria formed resting stages. Aggregate stability increased rapidly as casts age, probably due to increasing amounts of fungal hyphae. Polysaccharide content of casts was much greater than that of soil, but did not vary with changes in stability. Total and mineral nitrogen levels of casts were greater than those of soil; the major part of the inorganic nitrogen occurred as ammonia which was rapidly converted to nitrate.

INTRODUCTION

Earthworms feeding on soil and plant material set in train microbiological changes in the ingested food that continue when the cast is formed. Most microbiological investigations on worm casts have been concerned with the number of micro-organisms present. Stockli (1928), Kollmannsperger (1952, 1956), Ruschmann (1953*a*) and Schultz & Felber (1956) all reported more organisms in the gut or casts of earthworms than in the surrounding soil. Teotia, Dudley & McCalla (1950) found more bacteria but fewer fungi in casts than in soil and Rommell (1935) and Nef (1957) suggested that the feeding habits of earthworms alter the balance of soil population in favour of bacteria, so producing mull rather than mor conditions. Contrary to the findings of most workers, Day (1950) with *Lumbricus terrestris* in pot experiments found no significant difference between the numbers of bacteria in casts and soil. The present work examines changes in the microbial status of casts as they age under field conditions.

METHODS

The casting habits of earthworms vary; some species form casts on the soil surface, others within the soil in cracks, and some use cast material to line their burrows. Of the common field species in Britain, *Allolobophora terrestris* (Sav.) forma *longa* (Ude) and *A. nocturna* (Evans) are the main surface-casting types. Other species may form surface casts at times, but the habit is not general.

Casts are built up progressively by the frequent addition of small amounts of fresh material, so that different parts of a large cast are of different ages. This fact

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was taken into account when making observations on ageing casts. The casts used were produced by *Allolobophora terrestris* forma *longa*; casts and soil came from Great Field IV at Rothamsted. An area was freed from cast material and thereafter fresh casts were collected each day. These were bulked in groups of five and left in marked positions in the field and collected for examination as required. All soil samples were obtained from an area close to where the casts were collected.

Counting methods and media. These were the same as previously described (Parle, 1963), and included the use of selective media for cellulolytic and chitinolytic organisms. Proteolytic organisms were counted on a gelatin medium containing 200 g. powdered gelatin in 1000 ml. water, sterilized by steaming on three successive days.

Yeasts were identified by the methods of Lodder & Kreger-van Rij (1952), from poured plates. Fungal hyphae were measured by the method of Jones & Mollison (1948) using a 45 × objective and 10 × eyepiece with a micrometer scale.

Respiration was studied in the Warburg apparatus, using a slurry of the material to overcome differences in moisture content in casts of different ages (Chase & Gray, 1957).

Total nitrogen was estimated by the Kjeldahl method and mineral nitrogen by the methods of Bremner & Shaw (1955).

Bacterial polysaccharides were extracted by the method of Forsyth (1947) as modified by Chesters, Attoe & Allen (1957).

Aggregate stability was measured by the sodium saturation technique of Emerson (1954).

RESULTS

Numbers of micro-organisms in fresh casts and their variation with age

The only work so far reported on the change in microbial numbers with cast age is by Stockli (1928) who found that the numbers increased over the first 7 days. In an attempt to investigate this further, counts were made on casts collected after 1, 3, 5 and 20 days.

The counts in Table 1 confirm previous work (Parle, 1963) which showed a large increase in the microbial population passing through the worm gut. Rather higher soil populations were found than in the earlier work, probably because the soil organic matter increased during autumn. As the casts aged, the recovery of yeasts and filamentous fungi increased and actinomycetes appeared to increase toward the end of the period; bacterial numbers remained high. Irregular results were obtained with the counts of cellulose decomposers, but these appeared to be more numerous in fresh casts than in soils. Chitinolytic and proteolytic bacteria were fairly abundant in soil and casts and tended to decrease as the casts aged (except for chitinolytic bacteria at 20 days).

Fungi in ageing casts. The most notable feature of the cast material was the large increase in the number of yeasts and filamentous fungi with age; such an increase has not been reported before.

The most commonly occurring species of yeasts from cast material were: *Cryptococcus diffluens* (Zach.); *C. laurentii* (Kuff.); *Candida humicola* (Daszewska); *Trichosporon pullulans* (Lidner). Less frequently were found: *Candida curvata* (Diddens et Lodder); *Debaryomyces hansenii* (Zopf.); *Trichosporon cutaneum* (de

Table 1. *Numbers of micro-organisms in soil and in casts of* Allolobophora terrestris

(Mean log numbers per g. dry weight.)

Yeasts	Fungi	Actino-mycetes	Bacteria	Cellulose decomposers	Chitin decomposers	Gelatin decomposers
			No. of micro-organisms in soil			
5·20 (±0·069)	6·18 (±0·093)	7·60 (±0·170)	8·88 (±0·167)	1·48 (±0·452)	7·61 (±0·175)	7·30 (±0·268)
			No. of micro-organisms in casts			
			Fresh			
5·69	6·68	8·10	10·38	4·06	7·85	6·69
			One-day			
5·77	7·13	9·88	10·70	1·39	7·73	7·45
			Three-day			
5·80	6·88	8·41	10·16	3·39	7·51	6·74
			Five-day			
5·87	6·83	7·69	10·58	1·74	7·13	6·95
			Twenty-day			
6·17 (±0·115)	7·11 (±0·155)	9·81 (±0·284)	10·81 (±0·279)	0·00 (±0·756)	8·04 (±0·293)	2·43 (±0·448)

Table 2. *Fungi from casts of different ages*

Fungi	Soil	Fresh cast	Age in days: 1	2	3	10	15	20	30	50
	Percentage distribution									
Expt. 1										
Absidia	—	—	3·7	3·7	3·7	8·6	3·7	—	—	—
Cephalosporium	5·6	14·8	9·3	7·4	9·2	7·4	5·6	5·6	5·6	4·8
Cladosporium	11·1	3·0	3·7	5·6	5·6	5·6	7·4	5·6	—	3·2
Fusarium	—	—	—	3·7	—	—	3·7	—	—	—
Gliocladium	7·4	7·4	16·7	1·8	11·1	13·0	—	3·7	14·8	16·0
Oidiodendron	1·8	35·1	25·9	37·0	40·7	46·3	37·0	40·7	37·0	40·0
Penicillium	46·3	14·8	22·2	13·0	7·6	7·4	16·7	22·2	14·8	16·0
Trichoderma	5·6	—	1·8	—	1·8	—	11·1	—	3·7	—
Sterile	13·0	8·5	9·3	7·4	9·2	—	14·7	7·4	14·8	12·8
Unidentified	9·3	13·0	7·4	20·4	11·1	14·7	—	14·8	9·3	7·2
Expt. 2										
Cephalosporium	—	13·8	—	5·0	—	3·8	7·7	—	—	—
Cladosporium	1·8	28·9	—	5·0	11·5	9·7	15·4	5·8	7·7	3·8
Diplococcium	—	—	—	30·8	26·9	40·4	15·4	18·5	23·1	30·9
Fusarium	6·1	—	—	—	—	—	—	—	—	—
Geotrichium	5·4	—	11·8	3·8	—	—	—	3·8	3·8	5·8
Gliocladium	6·1	14·6	6·1	7·7	—	—	15·4	—	19·3	11·5
Oidiodendron	—	—	—	—	—	3·8	—	—	—	—
Penicillium	34·2	28·9	28·6	11·5	26·9	24·6	30·7	23·1	23·1	17·4
Trichoderma	1·4	—	—	—	11·5	—	—	4·6	3·8	11·5
Verticillium	—	—	12·5	9·2	3·8	7·7	7·7	13·5	7·7	3·8
Sterile	20·0	13·8	23·2	15·5	9·7	10·0	7·7	13·5	3·8	11·5
Unidentified	25·0	—	17·8	11·5	9·7	—	—	17·3	7·7	3·8

Beurm, Gougherot et Vaucher), and *Rhodotorula glutinis* (Fres.). Too few isolates were made to show whether any species were selected as casts age. None of the species isolated ferments sugars except *D. hansenii* which ferments glucose and sucrose weakly. These results are in accord with those of di Menna in New Zealand (personal communication) who rarely finds fermenting yeast species in soil.

Martin, Anderson & Coates (1942) examined changes in fungal populations as organic matter decomposed and found that the predominant fungal species depended on the availability of the energy source. This might also be expected in ageing casts. Casts of different ages and produced at different periods were sampled and plated on Lochhead's soil extract agar containing 30 μg. aureomycin/ml. Fungi appearing on the plates were identified after incubation for 14 days. Results of two such experiments done at different times (Table 2) show the relative abundance of the various groups as a percentage of the total isolates from a single sample.

On the occasion of the first count in November there was a preponderance of one genus, Oidiodendron, in the cast material of all ages, although very few were isolated from the soil. Oidiodendron occurs in association with decaying wood and litter (Dennis & Wakefield, 1946; Smith, 1946; Tribe, 1957) but is not common in soil. On the occasion of the second counts, starting 2 months later, Oidiodendron was found only once and Penicillium and Diplococcium species predominated. This suggests that no particular group of fungi is selected in the cast, but that the composition of the fungi population differs from time to time, possibly depending on the flora of the plant material recently eaten by the worm. Neither Oidiodendron nor Diplococcium was recovered from litter collected from the experimental area, but Penicillium and Cladosporium species were common.

Table 3. *Length of fungal hyphae per g. dry weight of casts of* Allolobophora terrestris

Sample	Length (m./g.)	Sample	Length (m./g.)
Expt. 1		Expt. 2	
Soil	79·0 ± 16·6	Fresh cast	120·8 ± 14·9
Fresh cast	133·4 ± 7·6	2-day cast	158·1 ± 16·2
5-day cast	284·2 ± 11·8	5-day cast	151·4 ± 13·3
10-day cast	434·8 ± 15·2	10-day cast	273·1 ± 14·5
15-day cast	607·3 ± 29·0	15-day cast	384·8 ± 15·1
20-day cast	528·5 ± 25·4	20-day cast	182·1 ± 12·2
25-day cast	408·4 ± 19·0	25-day cast	248·9 ± 13·5
45-day cast	415·4 ± 18·3		

The lengths of fungal hyphae in casts and soil

In much of the work on soil fungi, and in all that on fungi associated with earthworms, the dilution plating technique has been used, in spite of serious objections to this method for quantitative work. Hinson (1954) and Warcup (1955, 1957) showed that in many soils most colonies arising on dilution plates come from spores and not from growing fungi. To obtain some measure of the true fungal content of casts, total lengths of fungal hyphae were measured in material of different ages. Lengths were corrected for dry weight of the sample and results of two separate experiments are given in Table 3.

Good reproducibility was obtained within samples probably because the fine nature of the cast allowed even dispersal. The length of fungal hyphae increased greatly with age over a period of 15 days after the casts were formed. Some of this increase came from germinating spores and considerable amounts of young deeply staining material were seen. After 15 days the hyphal length declined slowly, because the fungi disintegrated.

Changes in microbial activity of ageing casts

Where there are large fluctuations in bacterial numbers, soil respiration is a valuable index of microbial activity (Chase & Gray, 1957; Katznelson & Stevenson, 1956; Rovira & Greacen, 1957 and Drobnik, 1958). Approximately 1 g. net weight of material to be examined was weighed into a Warburg flask and 2 ml. distilled water added; 0·2 ml. 20 % KOH was placed in the centre well. Duplicate flasks were prepared for each sample and hourly readings were made for 8 hr. at 25°. Flasks were shaken at 120 strokes/min. All values were corrected for the dry weight of the sample. Results are shown in Fig. 1, where each point represents the average oxygen uptake of duplicate flasks over the 8 hr. period.

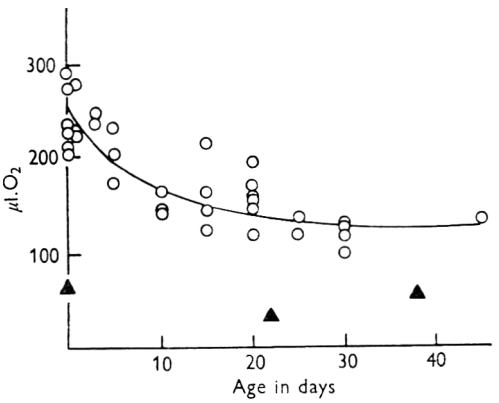

Fig. 1

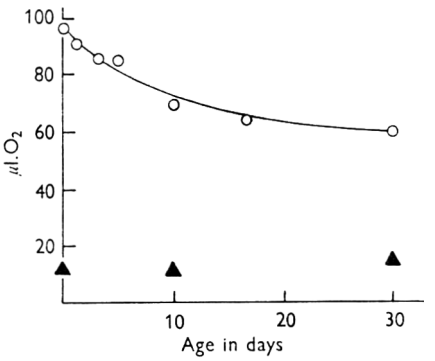

Fig. 2

Fig. 1. Oxygen uptake in 8 hr. by cast material of different ages. ○, Cast; ▲, soil.

Fig. 2. Oxygen uptake in 1 hr. by cast material of different ages in the presence of excess glucose. ○, Cast; ▲, soil.

The gradual fall in respiratory activity may be caused either by the number of respiring organisms decreasing or by the depletion of energy sources. To find which was primarily concerned, further experiments were made on the same cast material with added carbohydrate. After equilibration in the Warburg flask, 0·5 ml. of 0·005 M-glucose was added from a side arm to provide an excess energy source and readings were made at 10 min. intervals for 1 hr. This short time was chosen to minimize effects of microbial reproduction on oxygen uptake. Figure 2 shows that the pattern of oxygen uptake resembled that in the previous experiment, again indicating a decline in the number of actively respiring organisms with age of cast. This decline does not correspond with any of the population changes noted earlier and will be considered in the discussion.

Cellulose decomposition

The method used to count cellulolytic organisms gave irregular results and this group was studied further using the Warburg apparatus. Two sets of flasks were used, a control set and one containing 0·5 ml. of a cellulose suspension. Flasks were incubated with shaking for 96 hr., readings were taken for 12 hr. during the day and the manometer taps opened overnight.

Figure 3 shows ml. of oxygen used by casts of different ages. The number of cellulose decomposers fell as the casts aged, but activity was still greater than that of the soil after 40 days.

The nitrogen status of worm casts

Several workers have compared the chemical decomposition of worm casts with soil. It has been claimed that nitrification is enhanced in casts (Joshi & Kelkar, 1952; Day, 1950) and that casts contain more nitrate, amino acid and total nitrogen than soil (Lunt & Jacobson, 1944; Wittich, 1953; Shrinkhande & Pathak, 1951).

Total nitrogen in five sets of bulked casts is shown to be double that on two soils from the same area (Table 4).

Mineral nitrogen in casts was determined on 15–20 g. samples of material. The

Table 4. *Nitrogen contents of casts of* Allolobophora terrestris *and of soil*

	Sample	Total N (mg./g. dry wt.)
Cast	1	5·41
	2	5·52
	3	5·35
	4	5·99
	5	6·01
Soil	1	2·29
	2	2·34

Table 5. *Mineral nitrogen/g. dry weight of cast material*

Age (days)	Total mineral N (μg.)	NH_3-N (μg.)	NO_3-N (μg.)	NH_3-N (%)
0	282·3	272·8	9·5	96·6
	340·4	330·5	9·9	97·0
1	370·4	330·5	39·8	89·2
	404·3	362·5	41·8	89·7
2	256·7	239·1	17·5	93·2
	263·4	244·3	19·1	92·8
5	344·4	295·2	14·3	85·7
	339·4	294·9	13·1	86·9
7	278·7	222·3	56·4	79·8
	244·3	206·5	37·8	84·5
10	327·8	273·8	54·0	84·4
	262·1	237·6	24·5	90·7
20	134·6	82·3	52·3	61·1
	201·8	130·9	70·8	64·9

$CaCO_3$ excreted by the worm interfered with the determination and excess CO_2 was therefore first removed by placing soil extracts in a vacuum desiccator for 1 hr. before analysis; any loss of volume was made up with fresh extracting solution.

Table 5 shows the changes in amounts of nitrate and ammonia in casts of different ages. The percentage of total mineral nitrogen occurring as ammonia is also given. Each value is the average of duplicate analyses in different sets of casts. Because much cast material was required for each analysis the figures in the table represent the analyses on different samples collected on consecutive days. The results show a steady decline in content of ammonium nitrogen and an increase in nitrate. Nitrification begins immediately, as would be expected with such high levels of ammonia in the soil, much of the nitrogen lost from the cast during the 20-day period may have been nitrate.

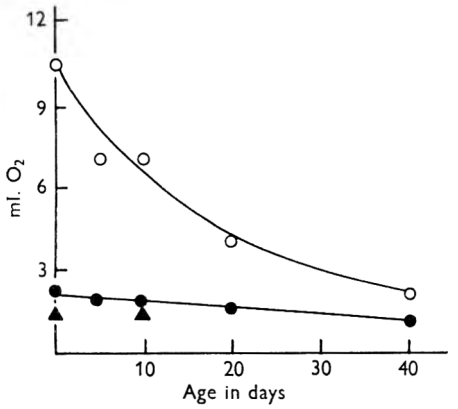

Fig. 3

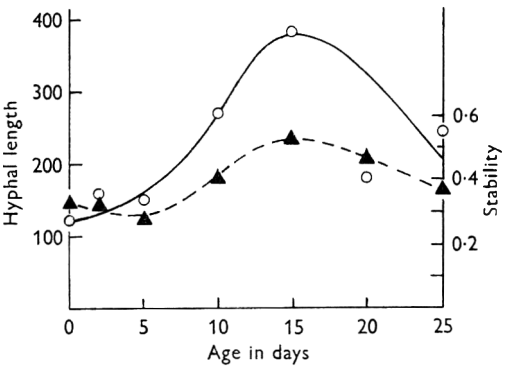

Fig. 4

Fig. 3. Oxygen uptake by casts of different ages in 96 hr. with cellulose as substrate. ○, Cellulose; ●, control; ▲, soil.

Fig. 4. Length of hyphae in m./g. and aggregate stability in ageing casts. Stability determined by ratio of permeability before and after sodium saturation. ○, Hyphae; ▲, stability.

The structural stability of casts

Fungi increase greatly in ageing casts and to investigate their possible stabilizing role, measurements of aggregate stability were made on casts of different ages, containing different amounts of fungal hyphae. Fungi are claimed by Martin & Waksman (1940) and McCalla (1945) to stabilize soil crumbs. Each set of measurements required approximately 15 g. of 1–2 mm. air-dried crumbs. To provide these, 60–80 g. wet weight of casts were collected daily and aged under normal field conditions. When required the samples were air-dried to constant weight, carefully broken and sieved to obtain the 1–2 mm. fraction. Three 5 g. samples were used to measure stability and some of the remaining material was used to prepare Jones & Mollison slides.

Figure 4 shows that stability alters as the casts age, rising to a peak at 15 days and then diminishing. The changes in stability are clearly correlated with length of fungal hyphae, indicating that the fungal content of casts largely determines their structural stability.

Bacterial polysaccharides and cast stability

Swaby (1948) and others suggested that bacterial gums play a part in increasing the aggregate stability of worm casts, and an attempt was made to examine this factor. Casts were collected from the same area and aged in the same way as for the previous experiment; 25 g. wet weight of casts was treated to extract polysaccharides which were then freed from inorganic impurities, dried at 50° and weighed.

Table 6 shows that much more polysaccharide was extracted from casts than from soil. The amounts of polysaccharide extracted at different times, however, were not correlated with cast stability. The 30-day-old cast may have lost polysaccharide because of decomposition by micro-organisms.

Table 6. *Weight of polysaccharide extracted/g. dry wt. of cast and percentage carbon in extracted material*

Cast age in days	Polysaccharide (mg./g. sample)	Carbon %
0	4·9	34·9
2	5·2	37·9
5	5·5	38·2
10	3·8	36·2
15	5·5	39·2
20	4·9	—
30	1·4	40·0
Soil	0·75	—

DISCUSSION

The high number of actinomycetes and bacteria in casts confirm earlier work, but these showed no consistent changes as the casts aged. The decline in microbial respiration with increasing age of casts, while microbial counts remained unchanged, suggests that the bacteria and actinomycetes formed resting stages. Schultz & Felber (1956) considered that bacteria in casts occurred mainly as spores. Counts and direct measurements showed that the fungal population increased greatly as casts aged a result contrary to those of Teotia *et al.* (1950) and Swaby (1949). Some reports (Rommell, 1935, and Nef, 1957) suggest that earthworms induce a predominantly bacterial population in soil by destroying fungal hyphae. My results do not accord with this view. The number of fungi found in fresh casts was not significantly different from the number in soil. Germinating fungal spores were commonly seen in fresh casts but only rarely in soil. The fungistatic factor found in soil by Jackson (1958) may not occur in casts and so allow spores to germinate. Although the stimulation of fungal growth was brief, lasting for about 15 days, the continuous ejection of cast material by worms would probably permanently increase the activity of the soil fungal population. The methods used do not show that worm activity favours the selection of any particular group of fungi, but more detailed examination is required.

Freshly formed casts are already stable. Such casts contain much polysaccharide which may play some part in stabilizing the aggregate. The suggestion by Swaby (1948) and others that such gums are water-soluble and will have little effect on aggregation is questionable, because little is known of their properties in soil and

they may be altered substantially by the extraction procedure; more gum is extracted by sodium hydroxide than by water. As the casts age, the stability of the 1–2 mm. fraction increases; the length of fungal hyphae also increases, and the increased stability may come from mechanical strengthening by fungi.

Results confirm that cast material contains more nitrogen than the surrounding soil, which is readily understandable when the selective feeding habits of worms are considered. Nye (1955) showed that when worms are kept in pots and all the nitrogen accounted for, their activity does not increase total nitrogen of the system. However, it is evident from analyses of mineral nitrogen in casts that worms change the character of ingested nitrogen, and this is probably important in the nitrogen cycle. Ammonia in fresh casts forms about 96 % of the extractable mineral nitrogen and this is rapidly converted to nitrate. Barley & Jennings (1959) showed that *Allolobophora caliginosa* increased available nitrogen by 6 %. With such high levels of mineral nitrogen as occur in casts there is little likelihood of atmospheric nitrogen fixation, and the antibiotic activity of Nocardia against Azotobacter noted by Ruschmann (1953*a*, *b*) has little significance. Moisture conditions under which worms work seldom favour the action of denitrifying organisms (Bremner & Shaw, 1958). The major contribution of earthworms in the nitrogen cycle in soil appears to be to increase the rate of mineralization of organic nitrogen.

In general, the value of earthworms in soil seems to lie in the fact that they increase the rate at which soil organic matter breaks down and in so doing may improve soil physical conditions for plant growth.

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The Effects of Two Quaternary Ammonium Compounds on Citric Acid and Sterol Synthesis in *Aspergillus niger*

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SUMMARY

A mono-quaternary amine, cetyltrimethylammonium bromide (Cetrimide), and a bisquaternary diamine, triclobisonium chloride (Triburon), were examined for their action on *Aspergillus niger*. Cetrimide, at 30 μg./ml. inhibited growth, but the organism was not inhibited by as much as 1·28 mg. Triburon/ml. Cetrimide at 20 μg./ml. inhibited citric acid accumulation. Triburon, at concentrations up to 160 μg./ml., increased the citric acid concentration, the maximum yield being at 40 μg. Triburon/ml.; at higher concentrations of Triburon there was an inhibition of citric acid production. The effect of Triburon was still perceptible in the presence of ferric ions. Cetrimide and Triburon brought about an initial increase in sterol concentration, followed by a decrease at higher concentrations of these compounds. The effects of these compounds on citric acid production by preformed mycelia were similar to the effect on cultures growing from spores.

INTRODUCTION

The use of quaternary ammonium compounds as topical antibacterial substances has become increasingly popular in recent times. A new class of these compounds, belonging to the bisquaternary group, has shown promise as systemic therapeutic agents as well (Schnitzer, Grunberg & DeLorenzo, 1960). Quaternary ammonium compounds are known to be comparatively innocuous to spore-forming organisms. A preliminary study in our laboratory showed a monoquaternary ammonium compound (cetyltrimethylammonium bromide, Cetrimide) to be highly inhibitory to the growth of *Aspergillus niger*, whereas a bisquaternary diamine (triclobisonium chloride, Triburon) was completely ineffective. The present paper deals with the effects of the two compounds on the growth and some aspects of the metabolism of *A. niger*.

METHODS

Triclobisonium chloride, *N*,*N*′-bis[1-methyl-3-(2,2,6,-trimethylcyclohexyl)propyl]-*N*,*N*′-dimethyl-1,6-hexanediamine bis(methochloride), (Triburon), was a gift from Hoffmann-La Roche, Inc., Basle (see Schnitzer, Grunberg, DeLorenzo & Bagdon, 1959). Cetrimide (cetyltrimethylammonium bromide) was the gift of Imperial Chemical Industries, Ltd.

Organism. The citric acid-accumulating strain of *Aspergillus niger* (Wisc. 72–4) was used, and was maintained by monthly subculture on potato glucose agar slopes.

Growth experiments. The organism was grown in 100 ml. conical flasks containing 10 ml. of the basal medium, which contained (g./l.); glucose, 150; NH_4NO_3, 2·5;

KH_2PO_4, 2·5; $MgSO_4.7H_2O$, 0·25; $MnSO_4.4H_2O$, 0·1; and $ZnSO_4.7H_2O$, 1·25 mg./l.; dissolved in triple glass-distilled water, and adjusted to pH 2·5 with HCl. The flasks were plugged with cottonwool and sterilized by autoclaving at 115° for 15 min. After cooling they were inoculated with a spore suspension from a 7-day slope culture. The flasks were incubated at 30° without shaking for the specified periods. After killing the organism by autoclaving, the mycelial mats were taken out, washed under the tap to remove spores, squeezed between filter-paper sheets and dried at 100°. The dry weight of the mycelium was used as a measure of growth.

Addition of the inhibitors. Cetrimide and Triburon were used as aqueous solutions and were either autoclaved in the basal medium, or sterilized separately by Seitz filtration and then added aseptically to previously autoclaved basal medium. Both methods gave identical results.

Experiments with resting mycelia of Aspergillus niger. The replacement technique used by Chughtai & Walker (1954) was adopted with minor modifications. The organism was initially grown for 4 days in 10 ml. lots of basal medium in 100 ml. conical flasks. On the 4th day, the culture medium was withdrawn and replaced aseptically with an equal volume of sterile basal medium without glucose. The flasks were then incubated overnight to make the mycelia use up any adsorbed glucose. The liquid in the flasks was again withdrawn and replaced with 10 ml. of 5% glucose solution, with or without the addition of the inhibitors as desired, and the flasks incubated at 30° for 24 hr.

Estimation of total acid and citric acid. Total acid was determined by titration of the culture medium against 0·1 N-NaOH to pH 8·0. Citric acid was estimated by the colorimetric method of Saffran & Denstedt (1948).

Estimation of sterol. The dry mycelium was repeatedly extracted by grinding with acetone+ethanol (1+1 by vol.) mixture. Sterols were estimated in the extracts by the method of Vahouny, Mayer, Roe & Treadwell (1960). Pure ergosterol, freshly recrystallized from ethyl acetate and dried *in vacuo* was used as a standard.

Estimation of glucose. Glucose consumption was measured by estimating the glucose remaining in the medium by the colour reaction with anthrone (Seifter, Dayton, Novic & Muntwyler, 1950).

Oxygen consumption of Aspergillus niger *mycelium in presence of Cetrimide or Triburon.* The organism was grown on the usual basal medium for 4 days, by which time uniformly thick mycelia formed. The mats were taken out, washed with distilled water, and pieces of equal weight (250 mg. fresh weight) cut out and placed in Warburg flasks of 16 ml. capacity, containing 3·0 ml. fresh basal medium, and different concentrations of the inhibitors. The gas phase was air, and oxygen consumption was measured by conventional methods (Umbreit, Burris & Stauffer, 1951) during 6 hr. at 30°. Oxygen consumption of mycelia grown in presence of the inhibitors was similarly measured.

RESULTS

The effect of Cetrimide and Triburon on growth, citric acid production and sterol concentration in Aspergillus niger

The incubation periods were 5 and 8 days. Under the conditions used, maximum acid production and growth was obtained at the end of 5 days; and when the

incubation was continued longer, the yield of citric acid and the mycelial weight both decreased. The results in Table 1 show that Cetrimide was inhibitory to the organism. Triburon, on the other hand, seemed to increase the mycelial weight. Sub-inhibitory concentrations of Cetrimide increased the sterol content two- to threefold, but had practically no effect on the formation of citric acid. Triburon produced similar, but less pronounced increases in sterol concentration. Intermediate concentrations of Triburon more than double citric acid formation. At higher concentrations, Triburon inhibited citric acid formation.

Table 1. *The effect of Cetrimide and Triburon on growth, total acid, citric acid and sterol of* Aspergillus niger

The average of ten separate experiments, each in triplicate, is presented below.

Additions to medium	Dry weight mycelium (mg.)		Total acid (ml. 0·1 N-NaOH)		Citric acid (mg./flask)		Sterol content of mycelium (μg./100 mg. dry mycelium)	
(μg./ml.)	5 days	8 days	5 days	8 days	5 days	8 days	5 days	8 days
None	222	169	39·6	33·5	282	239	292	325
Cetrimide								
0·8	223	172	38·9	32·8	282	244	342	406
1·6	220	172	37·8	34·0	276	236	618	687
3·2	215	175	38·8	33·3	280	239	698	776
6·4	226	169	38·7	32·8	285	242	788	877
10·0	209	170	35·6	30·1	269	249	705	784
12·8	198	168	34·0	29·8	260	235	458	510
20·0	179	174	2·8	23·3	20	165	179	198
30·0	No growth		—	—	—	—	—	—
Triburon								
0·8	223	174	53·0	44·8	377	320	343	382
1·6	225	169	57·9	48·9	410	349	389	435
3·2	223	172	75·1	64·9	544	465	551	606
6·4	223	175	90·5	76·6	646	550	455	507
12·8	225	170	106·8	88·7	758	634	316	352
20·0	229	171	110·4	90·6	789	646	297	324
40·0	233	176	120·0	95·9	855	682	292	325
80·0	259	198	102·1	86·1	726	613	279	311
160·0	282	222	89·6	82·4	631	586	265	307
320·0	288	254	39·2	31·2	275	216	247	309
640·0	285	280	31·6	31·5	225	225	241	303
1280·0	283	282	3·7	31·4	26	220	219	309

Glucose consumption was also studied in the same flasks by estimating the residual glucose; these results are presented in Table 2, including the yields of citric acid on the basis of glucose consumed. No significant effect of either substance on glucose consumption was observed, except for the inhibition produced by the highest concentrations of Cetrimide; in this case only the fermentation period of 5 days was studied.

Effect of Triburon on citric acid production in the presence of ferric ion

Since the effect of Triburon on citric acid production (Table 1) somewhat resembles the effect of ferric ion (see Perlman, Dorrell & Johnson, 1946; Shu & Johnson,

Table 2. *The effect of Cetrimide and Triburon on glucose consumption and yield of citric acid by* Aspergillus niger, *growing from a spore inoculum*

The results presented are the average of ten separate experiments. The fermentation period was 5 days.

Additions to basal medium (μg./ml.)	Glucose consumed (%)	Yield of citric acid (g./100 g. glucose consumed)
None	97·1	19·4
Cetrimide		
0·8	96·8	19·5
1·6	97·2	18·9
3·2	98·4	19·0
6·4	97·9	19·4
10·0	97·5	18·4
12·8	88·3	19·6
20·0	60·1	22·2
Triburon		
0·8	98·2	25·6
1·6	98·0	27·9
3·2	97·4	37·2
6·4	97·8	44·0
12·8	98·4	51·4
20·0	97·9	53·7
40·0	98·2	58·1
80·0	98·3	49·2
160·0	97·9	43·0
320·0	98·4	18·6
640·0	98·1	15·3
1280·0	98·1	1·8

Table 3. *The effect of Triburon on citric acid production by* Aspergillus niger, *in the presence of ferric ion*

The results presented are the average of eight separate experiments. The fermentation period was 5 days.

Triburon added to medium (μg./ml.)	Dry weight of mycelium (mg.)		Citric acid produced (mg./flask)		Sterol content of mycelium (μg./100 mg. dry weight)	
	Fe^{3+} 1 mg./l.	Fe^{3+} 15 mg./l.	Fe^{3+} 1 mg./l.	Fe^{3+} 15 mg./l.	Fe^{3+} 1 mg./l.	Fe^{3+} 15 mg./l.
0·0	225	223	542	72	305	294
0·8	223	223	694	92	347	339
1·6	226	226	760	100	407	401
3·2	222	224	822	109	575	553
6·4	219	225	859	114	462	450
12·8	224	220	889	118	316	322
20·0	230	225	952	127	290	296
40·0	234	237	1066	144	289	286
80·0	260	255	862	117	270	272
160·0	279	283	848	109	268	259
320·0	284	282	528	69	255	247
640·0	284	289	506	65	248	247
1280·0	289	280	34	5	224	210

1948) in so far as the yield of citric acid increased at first with increasing concentrations of Triburon, and then sharply decreased almost to zero, we examined whether this effect would persist in the presence of ferric ion. Two concentrations of iron were used, one being optimum for citric acid accumulation, and the other causing about 75 % inhibition of citric acid accumulation. These concentrations were determined in a separate experiment, and agreed with those reported by Shu & Johnson (1948). It was found that the effects of Triburon did persist in the two concentrations of iron, as shown in Table 3. The consumption of glucose was the same in the presence of iron as in its absence, and hence the highest amount of citric acid produced under the combined action of Triburon and iron represents a yield of about 72 % on the basis of glucose consumed.

Table 4. *The effect of Cetrimide and Triburon on glucose consumption and citric acid synthesis by preformed mycelia of* Aspergillus niger

The mycelia were incubated at 30° for 24 hr. in 5 % glucose solution. The results presented are the average of ten separate experiments.

Additions to medium (μg./ml.)	Glucose consumed (% initial concentration)	Citric acid produced (mg./flask)
None	53	45
Cetrimide		
4·0	54	47
8·0	52	45
12·0	55	44
16·0	50	46
20·0	27	22
40·0	1	0
Triburon		
4·0	54	45
8·0	53	57
12·0	54	74
16·0	50	102
20·0	51	154
40·0	54	98
80·0	52	46
160·0	50	28
320·0	52	7
640·0	14	0

Effect of Cetrimide and Triburon on citric acid synthesis and glucose utilization by preformed mycelium of Aspergillus niger

Mycelia were incubated at 30° for 24 hr. after the addition of the replacement medium and different concentrations of the inhibitors. No measurable growth took place during this period. Citric acid and glucose were estimated in the culture fluid; the results are shown in Table 4. Intermediate concentrations of Triburon were found to stimulate citric acid production, whereas Cetrimide had no such effect.

Oxygen consumption of Aspergillus niger *mycelium under the influence of Cetrimide and Triburon*

The inhibitors, at the concentrations used (0·4 mg. Cetrimide/ml.; 2·0 mg. Triburon/ml.), did not significantly affect oxygen consumption of *Aspergillus niger* mycelium. Oxygen consumption of mycelia grown in the presence of the two substances also did not differ significantly from that of normal mycelium.

DISCUSSION

The wide difference in the potencies of the two quaternary amines as inhibitors of *Aspergillus niger* is surprising. We have found (unpublished) that Cetrimide and Triburon are almost equally efficient (on a weight basis) in inhibiting the growth of such diverse organisms as *Saccharomyces cerevisiae*, *Torula utilis*, several lactic acid bacteria, *Escherichia coli*, *Staphylococcus aureus* and *Streptomyces griseus*. Further, no alteration in the sterol concentration was caused by the two inhibitors in such of the above organisms as contain sterol. The toxicity of both the quaternary amines for micro-organisms, including *A. niger*, increases with increasing pH value of the medium (unpublished work), whereas their detergent properties would decrease. Besides, the minimum concentration of Cetrimide required for micelle-formation, which is considered to be essential for detergent action, appears to be several times higher than the concentration at which inhibitory action becomes manifest. Hence, it seems reasonable to assume that the differences in the properties of the two quaternary amines cannot be attributed to the lack of detergent properties of the diamine Triburon.

Yields of citric acid corresponding to 70 % of glucose consumed have been reported in submerged as well as surface fermentation (Shu & Johnson, 1948; Perlman, Dorrell & Johnson, 1946). However, we did not obtain such high yields in our usual basal medium. The high yields obtained in the presence of suitable concentrations of Triburon, taken in conjunction with the lack of effect of the compound on glucose consumption, makes it look as though Triburon specifically alters glucose metabolism in favour of citric acid accumulation. Triburon causes alterations in the amounts of citric acid and of sterol, the effects being qualitatively similar. This would suggest that Triburon affects the synthesis or utilization of a common precursor, such as acetate, if it be assumed that most of the citric acid in *Aspergillus niger* is formed via the tricarboxylic acid cycle (see Ramakrishnan, Steel & Lentz, 1955; Bomstein & Johnson, 1952). However, it may be noted that Cetrimide, while greatly increasing the concentration of sterol, had no significant effect on citric acid synthesis. The data on oxygen consumption and glucose utilization suggest that effects of the inhibitors on these functions would not account for the observed effects on citric acid accumulation. The persistence of the influence of Triburon on citric acid production in the presence of ferric ion shows that the effects of this bisquaternary diamine and of the metal are independent of each other.

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The Haemagglutination Inhibitor in Edible Bird-Nest: its Biological and Physical Properties

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SUMMARY

This paper describes an investigation into the degree of homogeneity of the substance termed collocalia mucoid obtained from edible bird-nest. This material is a potent inhibitor of influenza virus haemagglutination and additional information is given here regarding its ability to neutralize infectivity. The crude material obtained by simple extraction was found to contain three components differing in molecular size and biological activity. Some separation of these was obtained by means of preparative ultracentrifugation. The slowest moving component differed from the others not only physically, but also in having much less inhibitor activity.

INTRODUCTION

Various animal sera and secretions contain substances which are able to inhibit haemagglutination by influenza viruses. These inhibitors have been divided into α and β types on the basis of their thermostability, their sensitivity to *Vibrio cholerae* neuraminidase, and the strain of influenza virus most susceptible to their action (Smith, Westwood & Belyavin, 1951). In more recent years a third class of inhibitor present in certain animal sera and characterized by its specific activity against A2 virus strains has been described (Shimojo, Sugiura, Akao & Enomoto, 1959; Cohen & Belyavin, 1959). This third type of inhibitor as it occurs in horse serum has been shown to be a potent infectivity-neutralizing substance in addition to its effectiveness as a simple haemagglutination inhibitor (Cohen & Belyavin, 1959). Other naturally occurring neutralizing inhibitors have been described, notably urinary mucoprotein (Tamm & Horsfall, 1952). The great variation in biological activity shown by these substances, both with regard to the type of virus predominantly inhibited and their ability to neutralize infectivity as well as haemagglutination, makes the chemical structure of these materials of great interest and importance.

Some work attempting to relate chemical structure to biological properties has already been published (Gottschalk & Fazekas de St Groth, 1960). Investigations in this direction are largely dependent upon the isolation of these inhibitors in a chemically homogeneous form. This is a technically difficult and time-consuming process where isolation of active inhibitor from crude serum is concerned. It is therefore of great interest to find other inhibitory substances present in fairly simple biological secretions from which they can be fractionated in a relatively pure form with comparative ease. Amongst these is a substance obtained from edible bird-nest

by Howe, Lee & Rose (1960) and called by them collocalia mucoid. It was decided to include this substance in a study of various inhibitors, but as a preliminary to the detailed investigation of the biological and chemical properties of collocalia mucoid it was necessary to establish the degree of homogeneity of material obtained by the method of Howe, Lee & Rose (1961). The results reported in this paper are concerned mainly with this question of homogeneity, but additional information is presented about the inhibitor properties of the material and in particular its ability to neutralize active virus.

METHODS

Nitrogen estimations. The nitrogen content was determined by the method of Paul (1958) with certain modifications. Test material was digested for 40 min. with 0·2 ml. of 50% H_2SO_4 containing 1% selenium dioxide. After digestion the tubes were cooled and 1·75 ml. of double-distilled water added followed by 5 ml. of alkaline Nessler solution. The tubes were left to stand for 20 min. and the intensity of the resulting colour read in the Unicam S.P. 500 spectrophotometer at 490 mμ using a 1 cm. optical cell. Samples of a standard solution of $(NH_4)_2SO_4$ containing 20 μg. N/0·5 ml. were included as controls in every test. As the extinction of nesslerized digest was linear with N concentration within a range of 5–80 μg. N/0·5 ml., it was usual to dilute the material so that its nitrogen content fell within these limits. When necessary a rough preliminary test was carried out to give an idea of the dilution required.

Sialic acid (*N*-acetylneuraminic acid). The thiobarbituric acid method of Warren (1959) was used. The method was standardized by comparing the results obtained with a purified mucopolysaccharide from ovarian cyst fluid (Pusztai & Morgan, 1961), containing an accurately known amount of sialic acid. As reported originally by Warren the extinction of the chromophore at 549 mμ was linear over the range 0·01–0·05 μmole.

Chromatography on calcium phosphate. The method of calcium phosphate chromatography used was that described by Tiselius, Hjertew & Levin (1956). The test material was adsorbed to Brushite columns at pH 8 in 0·01 M-phosphate buffer; gradient elutions were then performed with phosphate buffers of increasing molarity but constant pH value. Samples (10 ml.) were collected and tested for protein by measuring their extinction value at 280 mμ and for their inhibitor content against heated ROB (B type) virus.

DEAE-cellulose chromatography. Columns of DEAE-cellulose anionic form (Whatman DE 50 powder) were prepared according to the method of Sober, Gutter, Wycoff & Peterson (1956). The test material was adsorbed on to the columns at pH 6·8 and gradient elutions performed with various solutions as indicated in the text. The quantities of eluting buffers employed were adjusted so that small amounts of test material could be used. The volumes of fluid in both the mixing vessel and the charging vessel were each 50 ml. Samples of 3 ml. were collected and their protein content estimated by measuring their extinction values at 28 mμ.

Ultracentrifugation. Ultracentrifugation studies were carried out on an air-driven ultracentrifuge of the Beam and Pickels type, and also on a Spinco Model E. Material for analysis was taken up in 0·05 M-phosphate buffer and sedimentation

constants were calculated for water at 20° and extrapolated to zero concentration. We are very glad to acknowledge here our great indebtedness to Professor Baldwin and Dr E. Crook of the Biochemistry Department, University College London, and Dr K. Sanders of the M.R.C. Virus Research Unit, Carshalton, for placing their ultracentrifuge facilities so generously at our disposal.

Electrophoresis. Electrophoresis studies were carried out for us by Dr M. Rosemeyer of the Biochemistry Department, University College London, with a standard Tiselius apparatus. The details of the conditions used are given later.

Viruses. Three prototype influenza strains regularly used in this laboratory for the detection of different types of inhibitory activity were chosen. For detecting β activity, strain ASH, a representative A 1 strain, isolated in 1953 was used. The type B strain ROB isolated in this laboratory in 1955 was used after heating at 56° for 30 min. for detecting α activity. In additional tests the A 2 strain A/Singapore w was included to test for γ activity (Cohen & Belyavin, 1959). From time to time other strains were used as indicated. Each strain was maintained by allantoic passage in chick embryos. Infected allantoic fluids were prepared from seed viruses stored at −65° by inoculation of 10- to 12-day embryos with 0·2 ml. of seed diluted 10^{-3} in nutrient broth containing penicillin 2500 units/ml., and streptomycin 3000 μg./ml. After incubation at 35° for 72 hr. the eggs were chilled overnight and allantoic fluids harvested, pooled and stored at 4° until required.

Haemagglutination (HA) and haemagglutination inhibition (HI) titrations. Titrations were done by the photoelectric densitometer method of Hirst & Pickels (1942) modified by Belyavin, Westwood, Please & Smith (1951). Titres were obtained from densitometer readings by the use of a nomogram (Lim, 1954) for 50% end-point interpolations.

Neutralization tests in eggs. Serial dilutions of the test material in penicillin+streptomycin broth were mixed with equal volumes of the virus diluted to contain 1000 EID 50 (egg infective doses) in 0·2 ml., and the mixtures held at 37° for 30 min. Groups of six 11-day chick embryos were inoculated with 0·2 ml. volumes of each mixture. In parallel, the virus challenge dose was checked by a standard infectivity titration. After incubation for 72 hr. the eggs were chilled, harvested and each allantoic fluid tested for virus by haemagglutination in WHO plastic trays; 50% infectivity end-points were calculated by the method of moving averages (Thomson, 1947).

Neuraminidase (receptor destroying enzyme; RDE) prepared from *Vibrio cholerae* by the method of Burnet & Stone (1947) was purified by one cycle of adsorption and elution with human group O red cells.

Treatment of inhibitor with neuraminidase. Solutions 0·2% of lyophilized inhibitor were prepared in calcium acetate buffered saline (pH 6·2). These were diluted with an equal volume of RDE (neuraminidase) diluted one in two in calcium acetate buffered saline. The mixtures were held at 37° for 4 hr. and then at 56° for 30 min. to inactivate the residual active enzyme. Controls consisted of (*a*) the 0·2% solution of inhibitor diluted one in two in calcium acetate buffered saline, and heated in parallel with the test solutions, and (*b*) a similar dilution which was unheated.

Preparing collocalia mucoid. The method used for preparing collocalia mucoid was essentially as described by Howe, Lee & Rose (1961) except that the crude bird-nest material was soaked in cold water before extraction at 62°. This was done to

wash out any free sialic acid from the crude material. The crude bird-nest substance was purchased from a dealer and it was noted that two grades of material (referred to subsequently as first and second quality) were retailed. Comparative tests showed that there was a considerable difference in yield of inhibitory activity between the two grades and that they also differed in respect of the 'free' sialic acid content (Table 1). As a result care was taken to use only 'first grade' material for all

Table 1. *Comparison of sialic acid content and haemagglutination inhibition (HI) activity of distilled water extracts prepared from 'first' and 'second' quality edible bird-nest material*

Extractions performed for different times at 62° on a 10 % suspension of crude material.

Material		Total sialic acid (μg./ml.)	Free sialic acid (μg./ml.)	Combined sialic acid (μg./ml.)	HI titre v. heated ROB	Specific activity HI/μg. sialic acid combined
2nd quality	½ hr. extract	320	300	20	96	4·8
	1 hr. extract	420	300	120	250	2·1
	4 hr. extract	n.t.	n.t.	n.t.	425	—
1st quality	2 hr. washing*	n.t.	107	n.t.	461	—
	2 hr. extract	720	205	515	> 10,240	> 20·0
	4 hr. extract	3,360	303	3,057	> 10,240	> 3·0

* At room temperature; n.t. = not tested.

Table 2. *Haemagglutination inhibitor (HI) concentration in successive extracts of crude edible bird-nest at* 62°

Material*	HI titre v. heated ROB
1st extract	4150
2nd extract	2998
3rd extract	5263

* At a concentration of 2·5 %.

subsequent preparative work. It is interesting to note that the 'second grade' material was obviously different in appearance, being somewhat darker in colour, and also being evidently contaminated with feathers and other extraneous matter.

Repeated extractions of a given batch of material showed continued yield of inhibitor (Table 2) and multiple extractions were therefore adopted as a standard procedure. Extractions at a lower temperature yielded only very little inhibitor. The final method of preparation adopted was as follows. Powdered crude bird-nest (12 g.) was soaked in distilled water (200 ml.) for 2 hr. at room temperature on two successive occasions. The washings were discarded and the solid material then extracted at 62° three times in succession, each extraction lasting 4 hr., with 1000 ml. distilled water in the first and 500 ml. in each of the other extractions. Between extractions the solid material was separated by filtration through Whatman No. 1 filter paper on a Buchner funnel; finally the pooled extracts were lyophilized. The yield under these conditions was about 100 mg. lyophilized powder/g. crude bird-nest material extracted. The fluffy white material obtained on lyophilization

was initially readily soluble in water, but on storage some deterioration occurred. The substance became less soluble and there was a loss of specific inhibitory activity. Even the fresh material was easily precipitated from solution by the addition of electrolytes when these exceeded a certain concentration.

RESULTS

Homogeneity of crude collocalia mucoid

Calcium phosphate chromatography. Crude lyophilized extract (10–20 mg.) was made up in 10 ml. of 0·01 M-phosphate buffer (pH 8·0), and applied to calcium phosphate columns 10 × 3 cm. Gradient elution of preparations made from the

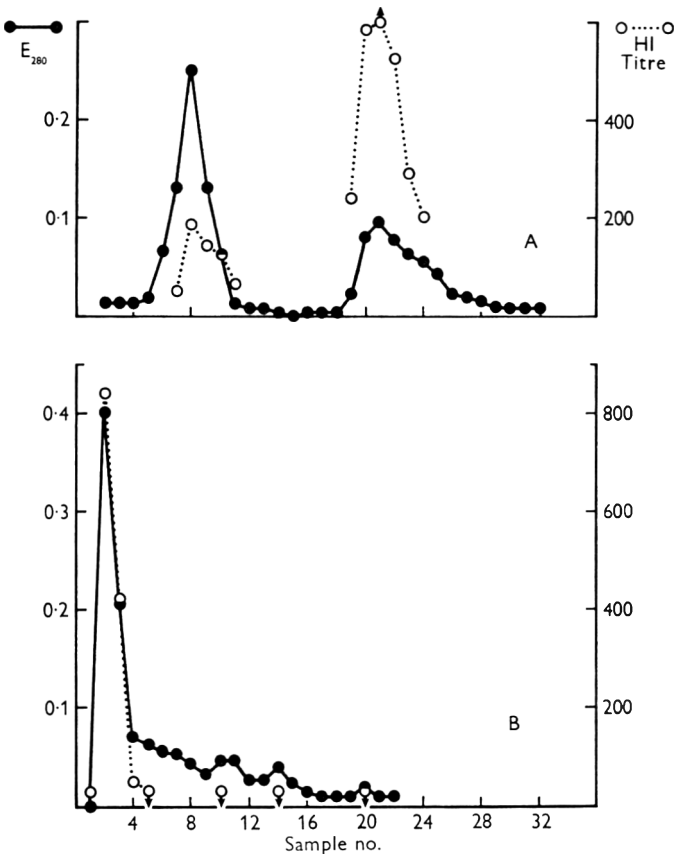

Fig. 1A. Calcium phosphate chromatography of a crude extract of collocalia mucoid showing separation into two components.

Fig. 1B. Calcium phosphate chromatography of crude collocalia mucoid showing the usual pattern obtained, consisting of a single component only.

original batch of bird-nest yielded a chromatographic pattern characterized by two clear-cut protein peaks (Fig. 1A). Inhibition tests showed that the second peak had a higher specific activity/mg. nitrogen than the leading peak. This was confirmed by stepwise elution of the same initially ophilized preparations, but every attempt to

concentrate and lyophilize the second active peak material led to the isolation of a completely insoluble and apparently denatured substance. Four consecutive extracts from the same initial batch of bird-nest material yielded this chromatographic pattern, irrespective of the calcium phosphate preparation used. All subsequently purchased batches of bird-nest material extracted by the procedure described above, however, have yielded a single dominant protein peak, with a corresponding inhibitory pattern (Fig. 1B). The specific inhibitory activity of this peak

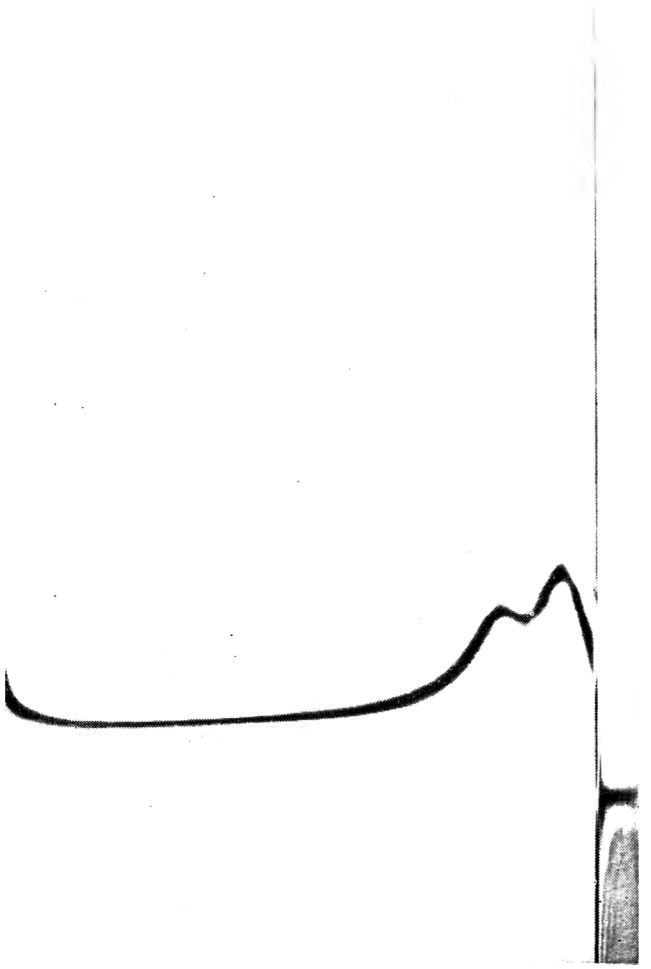

Fig. 2. Ultracentrifuge pattern of crude collocalia mucoid showing early separation of fast-moving (20S) component. (4 min. after reaching maximum speed—50,740 rev./min.) Sedimentation towards left.

was not markedly different either from the original starting material or from the first peak obtained in the earlier chromatograms. As this single peak was evidently due to material not adsorbing on the column, it was clear that no resolution of the crude material was being obtained by this system.

DEAE-cellulose chromatography. Since calcium phosphate did not resolve collocalia mucoid it was decided to try DEAE-cellulose columns. Work in this laboratory has indicated that Brushite columns are cationic and so it was considered possible that an anionic exchanger might affect resolution.

Crude collocalia mucoid (100 mg.) was dissolved in 10 ml. 0·01 M-phosphate buffer (pH 6·8), adsorbed on a column of DEAE-cellulose 1 cm. × 5 cm. containing 2·5 g. dry DEAE-cellulose. The following gradients were then used in succession: (1) to 0·02 M-phosphate buffer (pH 6·8); (2) to 0·05 M-NaH_2PO_4 + 0·05 M-NaCl; (3) to 0·1 M-NaCl; (4) to 0·2 M-NaCl. None of the adsorbed material was recovered in the effluent at any stage of the eluting gradients. Two explanations are possible. The collocalia mucoid might have been largely denatured by the electrolyte in the eluting buffers, and thus failed to penetrate the column. It is also possible that, due to its high negative charge, it formed a strong union with the positively charged DEAE-cellulose and thus remained adsorbed. As the method did not seem very promising, this technique was not pursued any further at this stage.

Analytical ultracentrifugation. Examination of the crude material in the analytical ultracentrifuge showed two well marked and easily separable peaks (Fig. 2). The ammonium sulphate precipitated material and the lyophilized concentrate of the single peak isolated chromatographically yielded a similar pattern. Later studies showed that with prolonged centrifugation at maximum speed the slow-moving component could be resolved into two further peaks. It was clear that at least three physically distinguishable components were present in the crude extract of collocalia mucoid and the sedimentation constants of these were calculated to be 1·4S, 4·7S and 20S, respectively (the 1·4S value is calculated from a run at one concentration only). Preliminary examination of the material obtained by low temperature extraction showed it to be comparable in sedimentation velocity to the 1·4S component.

Electrophoresis. The crude extract was studied in the Tiselius electrophoresis apparatus. Solutions were prepared in 2-amino-2-hydroxymethylpropane-1,3-diol (tris) + acetate buffer (pH 8·16, *I* 0·06 and in sodium acetate buffer pH 5·4, *I* 0·06; with the former solution a current of 1·7 mA was used, and with the latter a current of 2·6 mA. Owing to the relative insolubility of collocalia mucoid the concentrations achieved were not very great, and after about 3 hr. the effects of diffusion caused marked blurring of the migrating boundaries. The duration of the run therefore was confined to this period; but within this time only a single peak was seen and this migrated rapidly towards the anode. The rapid movement of the material towards the anode was consistent with the possession of a high negative charge from the presence of sialic acid groupings, as is known to be the case with red blood cells.

Attempts at fractionation

Ammonium sulphate precipitation. Precipitation with ammonium sulphate gave a dense white precipitate which was easily soluble in water and which contained all the activity of the crude extract. On ultracentrifugation the ammonium sulphate precipitated material still showed two peaks and the specific activity did not differ significantly from that of the original (Table 3). This was confirmed by the fact that both the ammonium sulphate precipitate and the original material each contained about 10% sialic acid, indicating no essential difference between them.

Ethanol fractionation. Ethanol precipitation was tried as a further means of purification and at 75% (v/v) ethanol a dense white precipitate was obtained containing a substantial amount of haemagglutination-inhibition activity. A formal experiment was conducted in which potassium acetate was added to the crude extract in different proportions as has been used for the precipitation of mucoid substances (Pusztai & Morgan, 1961). Ethanol was added at 4° and the precipitate, if any, separated by centrifugation and taken up in a volume of water equal to that of the original. It was then tested for HI activity and the results show (Table 4) that the specific activity of the ethanol precipitate was no greater than that of the original. Comparison of the specific activities of the control preparations at the different concentrations of potassium acetate shows the deleterious effect of this salt on collocalia mucoid. Subsequent work has shown that this material can be precipitated from solution by a variety of electrolytes. Even in the absence of salts, however, the ethanol precipitate was so easily denatured that concentration was impossible, thus precluding further tests at this stage.

Table 3. *Total nitrogen, haemagglutination inhibitor potency (HI) and specific activity of crude extracts and ammonium sulphate (AmS) precipitates prepared from different batches of bird-nest material (all compared as 0·1% solutions of lyophilized preparations)*

Batch of nest material	Preparation	Total nitrogen (μg./ml)	HI titre v. heated ROB	Specific activity HI/μg. N/ml.
A*	Crude extract	110	1856	17
	AmS ppt.	110	1385	14
B	AmS ppt. (1)	110	1033	9
	AmS ppt. (2)	110	1536	14
C	Crude extract (1)	110	1997	19
	Crude extract (2)	110	2200	20
	Crude extract (3)	110	1530	19
	AmS ppt. (1)	87	1530	17
	AmS ppt. (2)	88	973	11
D	Crude extract (1)	92	1011	11
	AmS ppt.	73	1120	15
	Crude extract (2)	87	1472	17

* The only preparation yielding two peaks on calcium phosphate chromatography.

Fractionation by ultracentrifugation. An attempt was made to separate the three physically different components of collocalia mucoid by using the Spinco Model L preparative ultracentrifuge. Solutions of the material were centrifuged at 100,000 *g* (number 40 rotor) for periods of 2 and 6 hr. Examination of the 2 hr. supernatant fluid in the analytical ultracentrifuge did not detect any residual fast-moving (20S) component. The sialic acid, nitrogen and inhibitor values of the 2 and 6 hr. supernatant fluids were then compared with those of the original material before centrifugation (Table 5). The significance of these results will be discussed later, but certain features may be noted here. The lower specific activity of both the original material and the supernatant fluid in one experiment (Expt. 5, Table 5) was the result of storage of the mucoid, as discussed previously. The most significant feature of the results

obtained is that after centrifuging for 6 hr. the residual supernatant fluid still contained about one-quarter to one-third of the nitrogen and sialic acid of the original, but the corresponding inhibitory activity had been decreased to less than one-tenth.

Table 4. *Results of ethanol precipitation of crude bird-nest extract and the effect of adding potassium acetate on the specific activity*

Ethanol concentration (v/v)	K acetate added (%)	HI titre v. heated ROB	μg. N/ml. (precipitate)	Specific activity HI/μg. N/ml.
25 %	0·5	< 80	17·5	< 5
50 %	0·5	< 80	16	< 5
75 %	0·5	966	80	12
Control	0·5	1120	90	12
25 %	1·0	120	40	3
50 %	1·0	320	58	5
75 %	1·0	720	85	9
Control	1·0	515	90	6
25 %	2·0	126	58	2
50 %	2·0	189	60	3
75 %	2·0	390	60	7
Control	2·0	890	115	8
Control	nil	1574	105	15

Table 5. *Results of centrifuging crude collocalia mucoid extracts at 100,000 g for different periods*

Expt.	Preparation	Concentration (%)	Time of centrifuging (hr.)	Total N (μg./ml.)	Total sialic acid (μg./ml.)	HI titre v. heated ROB	Specific activity HI/μg. N/ml.
1	A	0·2	0	n.t.	n.t.	650	—
			2	n.t.	n.t.	153	—
2	D	0·5	0	380	360	3850	11
			2	200	200	1930	10
3	D	0·1*	0	88	82	1538	17
		0·1*	2	74	72	1472	20
4	D	1	0	860	800	7885	9
			2	580	560	4480	8
			6	260	210	344	1·3
5	D	1	0	980	800	4070	4
			2	480	350	730	1·7
			6	240	250	90	0·4
6	C	1·5	0	1040	1050	20480	20
			2	460	510	3635	8
			7½	288	300	160	0·5

* Both the original material and centrifugal supernatant lyophilized and made up as 0·1 % solutions; n.t. = not tested.

Inhibitor activity

Crude extracts, ammonium sulphate precipitates and ultracentrifuge supernatant fluids were tested for haemagglutination inhibition activity against prototype virus strains. Our earliest preparations were tested against heated ROB (B)

Table 6. *'Spectrum' of inhibitory activity of collocalia mucoid*

Expt.	Type of material	Treatment of material	Inhibitory activity against nominal four agglutinating doses of virus							
			PR 8		ASII		ROB		A/Singapore W	
			Unheated	Heated	Unheated	Heated	Unheated	Heated	Unheated	Heated
1	Crude extract	Unheated	2,560	> 20,480	2,560	> 20,480	1,180	2,867	256	806
2	Crude extract	Treated RDE	n.t.	n.t.	560	106	119	< 80	114	108
		Heated control	n.t.	n.t.	3,560	5,120	919	2,700	1,930	870
3	AmS ppt.	Treated RDE	n.t.	n.t.	80	< 80	n.t.	< 80	< 80	n.t.
		Heated control	n.t.	n.t.	1,152	> 2,560	n.t.	1,760	230	n.t.
4	Crude extract	Treated RDE	n.t.	n.t.	< 80	< 80	< 80	< 80	< 80	< 80
		Heated control	n.t.	n.t.	410	998	640	1,150	483	280
		Unheated control	n.t.	n.t.	1,030	2,982	840	2,355	960	667

* AmS = ammonium sulphate; n.t. = not tested.

virus and, as already seen, were potent haemagglutination inhibitors of this strain. We also found that this activity was destroyed by neuraminidase with the accompanying release of sialic acid. It seemed therefore that this substance possessed classical α inhibitory activity. More extensive examination showed that it was active against a wide range of unheated virus strains. The results of these experiments are summarized in Table 6. The apparent loss of inhibitory activity of the collocalia mucoid when heated as a control preparation for the tests of neuraminidase sensitivity was found to be due to the presence of the acetate ions in the buffer used. It may be noted that collocalia mucoid is active against the heated and the unheated prototype viruses to varying degrees, maximal activity against an unheated strain being found with the A 1 strain ASH. The material left after centrifuging away the fast-moving 20 S component as described above was tested against the same virus strains (see Table 6) and exhibited a similar pattern of activity. Tests against selected strains suggested that this pattern of inhibitory activity against unheated virus was paralleled by the spectrum of neutralizing activity in ovo. It was found that maximal activity was exhibited against the type A 1 strain ASH, 1 μg. of the collocalia mucoid being sufficient to neutralize 1000 EID 50 of virus.

DISCUSSION

The data given here suggest that collocalia mucoid obtained by a simple process of extraction is not homogeneous, but the nature of its inhomogeneity is worthy of further discussion. The three peaks seen in the analytical ultracentrifuge suggest the presence of three substances with different molecular weights. All attempts to separate these by various chemical fractionation methods were unsuccessful, except in the earlier chromatographic studies when two components were separated, the second having a much greater specific activity than the first. However, no detailed study of this component could be made since it became inactivated on concentration. The full significance of these findings therefore remains obscure. The results of analytical ultracentrifugation indicated that some separation of the various components might be achieved on the preparative ultracentrifuge. It was shown subsequently that centrifugation of the crude material at 100,000 g for 2 hr. removed the bulk of the fast-moving (20 S) component as verified in the analytical ultracentrifuge, and left behind the slow-moving (1·4 S and 4·7 S) components. Examination of the freeze-dried material from the supernatant fluid showed that the nitrogen, sialic acid, and inhibitor values were equal to that of the whole; but more prolonged centrifugation for 6 hr., which must inevitably have isolated the slowest moving component, yielded a substance having a very low specific inhibitor activity. Thus it would appear that each of the three different molecular species possessed different biological activities. It was considered possible that the two slow-moving peaks resulted from a breakdown of a single substance, occurring under the influence of the centrifugal force, one fragment carrying the bulk of the activity. Against this hypothesis is the finding that low-temperature extraction of the original bird-nest material gave a substance with a very low inhibitory activity and which; on preliminary examination in the analytical ultracentrifuge appeared to have a low rate of sedimentation corresponding to the slowest moving peak seen in the crude extracts. It is likely from this that three physically (and probably biologically) different

substances are present in the original crude preparation. About the electrophoresis studies it is difficult to arrive at any firm conclusions at this stage, except to say that whatever the differences between the three physically different components already discussed, they do not appear to differ in respect of the charges carried by them.

The picture just presented of the inhomogeneity of collocalia mucoid is made more interesting by the nitrogen and sialic acid values of the centrifugally separated components. The ratio of these values was fairly constant, irrespective of whether the material examined was a crude extract, that resulting from attempts at chemical fractionation, or the components obtained by ultracentrifugation. This constant relationship suggests a common chemical structure independent of physical inhomogeneity and this may be the result of depolymerization of a single large molecule. However, this can only be proved when the various components have been isolated in pure form, and for this reason a full chemical investigation was not carried out at this stage. Howe *et al.* (1961) reported a detailed chemical analysis of collocalia mucoid but gave no indication about the degree of homogeneity of their material. In the absence of any specific information about this point, and in view of our own findings, their results cannot be properly assessed, particularly as regards the relationship between the inhibitor properties and the chemical structure of collocalia mucoid.

The sensitivity of collocalia mucoid haemagglutination inhibitor to the action of neuraminidase, and its activity against the haemagglutinin of heated type B virus strains, classes it by definition, as an α type inhibitor. It has been shown, however, to have a wide range of haemagglutination inhibiting action against other strains including the A1 strain ASH, in the unheated state. The ability of collocalia mucoid to inhibit the haemagglutinin of an unheated virus is paralleled by its active neutralization of infectivity of the corresponding strain. Thus both strain ASH, and A/Singapore are readily neutralized, whereas unheated ROB is only weakly so. The demonstrable inhomogeneity of collocalia mucoid might suggest that this wide spectrum of activity against strains other than the heated type B strain ROB was due to the presence of a mixture of inhibitory substances. This is clearly refuted, however, by the very similar range of haemagglutination inhibition shown by the supernatant fluid after centrifugation at 100,000 *g* for 2 hr., under which conditions the bulk of the 20S component was sedimented. Extending the period of centrifugation under these conditions to 6 hr. leaves a slowly sedimenting component which has very little inhibitory activity and thus presumably contributes little to the activity of the whole. It seems reasonable, therefore, to regard each of the distinguishable and biologically active molecular species of collocalia mucoid as carrying the same 'spectrum' of inhibitory activity; a conclusion further supported by the fact that the activity against all sensitive strains is more or less proportionately destroyed by neuraminidase.

The breadth of inhibitor 'spectrum' exhibited by this material is of some interest, when compared with the highly purified α type inhibitor prepared by Pusztai & Morgan (1961) from ovarian cyst fluid. This latter material is not active in the haemagglutination inhibition test against representative unheated type A, A1, A2, and B viruses, the whole of its inhibitory potency being directed against the heated (indicator) type B strains. Against such a strain it is as active, weight for weight, as

collocalia mucoid. There seems a clear indication here that differences in the range of inhibitory action observed amongst haemagglutination inhibitors of the α type may be dependent on variations of molecular structure and chemical constitution, and that the property of neuraminidase sensitivity defines a chemically heterogeneous group of substances. Comparative investigations by other workers has already produced results to suggest that this is probably so (Gottschalk & Fazekas de St Groth, 1960). The presence of a small molecular component in collocalia mucoid, which has a similar sialic acid/nitrogen ratio to that found for the other faster sedimenting components, but only about a tenth of their specific inhibitor activity, raises an interesting question regarding the importance of sialic acid to the α inhibitory activity of a mucoprotein molecule. An elucidation of this problem clearly awaits the separation and purification of the molecular components found in collocalia mucoid.

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The Genetic Analysis of Carbohydrate Utilization in *Aspergillus nidulans*

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SUMMARY

The isolation and genetic analysis of a new class of mutants of the mould *Aspergillus nidulans* is described. The mutants were detected by their inability to utilize specific carbohydrates as sole carbon source for growth. All of the mutants are recessive and analysis of 27 mutants has resulted in the description of 10 new loci concerned with the utilization of carbohydrates in the organism; the loci have been allocated to linkage groups and 6 were mapped meiotically. Two loci which control lactose utilization are not linked and neither are 5 loci which control the utilization of galactose. A series of sorbitol mutants is unusual in that they are all non-complementary when combined in heterokaryons, although certain pairs of mutants complement in the corresponding heterozygous diploids.

INTRODUCTION

It is a conspicuous omission in fungal genetics that although auxotrophic mutants have been isolated in many filamentous fungi and it is known that in general these fungi metabolize a wide range of carbohydrates, very few attempts to isolate mutants defective in carbohydrate utilization have been reported (Emerson, 1944; Landman, 1950). Mutants defective either in the ability to form specific carbohydrases or carbohydrate permeases (Cohen & Monod, 1957) may be identified by their failure to grow with particular carbohydrates as sole carbon source. The present paper describes the isolation and genetic analysis of mutants of this type in the homothallic ascomycete *Aspergillus nidulans*.

METHODS

Strains and media. The strains of *Aspergillus nidulans* used were from the stock held in the Department of Genetics of Glasgow University. Media and routine methods for *A. nidulans* described by Pontecorvo *et al.* (1953) were used. Minimal medium is a solution of inorganic salts, including nitrate and sulphate as nitrogen and sulphur sources, with 1 % (w/v) glucose as carbon source. Complete medium is a complex medium containing casein hydrolysate, yeast extract, some vitamins, a hydrolysate of yeast nucleic acid, and salts and glucose as in minimal medium. Basal medium, used in the isolation of the sugar mutants and in classifying strains by their ability to utilize different sugars for growth, is minimal medium without a carbon source. The media were solidified by adding 1·5 % (w/v) unwashed Difco agar. Sugars were sterilized separately by autoclaving (115° for 10 min.) in

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distilled water and were added to molten basal medium to a final concentration of 1 % (w/v). The sugars used were D-glucose, lactose and sucrose (all analytical reagents) and D-fructose (biochemical reagent) from British Drug Houses Ltd.; D-galactose, maltose and D-sorbitol (all bacteriological reagents) from T. Kerfoot, Vale of Bardsley, Lancs.

Isolation of mutants. The mutants were isolated by a replica plating technique (Roberts, 1959) following ultraviolet (u.v.)-irradiation. Suspensions of mainly separate conidia of *Aspergillus nidulans* strains *bi1*; *w3* (biotinless; white conidia) or *y2*; *pyro4* (yellow conidia; pyridoxinless), were exposed to u.v. radiation until the viable count was decreased to about 5 % of the original value; the suspensions were then diluted and plated on complete medium to yield 100 to 150 colonies on each plate. The plates were incubated until good sporulation had occurred, when conidia were replicated on to a series of seven plates. Five were test plates containing basal medium supplemented with either biotin or pyridoxin (required by either parent strain), and to each of which either galactose, lactose, maltose, sorbitol or sucrose had been added as sole source of carbon: the sixth was a control containing minimal medium (glucose) supplemented with either biotin or pyridoxin, and the seventh plate contained complete medium.

Auxotrophic mutants with a nutritional requirement in addition to that of the parent strain were detected by their failure to grow on any of the five test plates or on supplemented minimal medium, though growing on complete medium. Sugar mutants were identified by their failure to grow on one or more of the test plates although growing on supplemented minimal medium and on complete medium. Mutants were isolated from the complete medium plate, purified by single colony isolation and their phenotypes retested. Only strains clearly different from the wild-type in their growth on the relevant sugar (though growing as well as the parent strain on glucose) were retained for analysis. When more than one mutant was isolated from the same set of plates only one was retained unless they were clearly different phenotypically.

Genetic analysis of mutants. Phenotypically similar mutants were separated into functional groups by complementation tests (Pontecorvo, 1952*a*; 1958) made with the appropriate heterozygous diploids (Roper, 1952). Location of the mutants in the linkage maps of *Aspergillus nidulans* was achieved in two stages; first, identification of the linkage group into which a mutant fell and, secondly, location of the mutant in relation to other markers in that group. The first stage of location used the parasexual cycle (Pontecorvo, 1954) and the technique of mitotic haploidization (Pontecorvo, Tarr Gloor & Forbes, 1954; Pontecorvo & Käfer, 1958) combined with the use of 'tester strains' (Forbes, 1959) marked in each of the eight linkage groups of *A. nidulans* (Käfer, 1958; Elliott, 1960). The second stage of location used the sexual cycle and the technique of perithecium analysis (Hemmons, Pontecorvo & Bufton, 1953). Methods given by Mather (1951) were followed in the detection and estimation of linkage.

Markers used in the present work. The markers used in the analysis are listed at the bottom of Figs. 1 and 2; for further details see Pontecorvo *et al.* (1953) and Käfer (1958).

RESULTS

Carbohydrate utilization by the wild-type

A brief preliminary survey of the growth of *Aspergillus nidulans* on a variety of organic compounds as sole carbon source was made by use of an auxanographic technique (Pontecorvo, 1949). A thick suspension of wild-type conidia in basal medium was poured in a series of Petri dishes, and these incubated for 18 hr. at 37°. Crystals of the various compounds were then placed at points on the surface of the agar and the resulting growth compared after a further 24 hr. of incubation. Good growth was obtained with D-glucose, D-fructose, sucrose, maltose, cellobiose, acetate, glycerol and D-sorbitol; moderate growth with mannose, D-galactose, rhamnose, melibiose, lactose, trehalose, raffinose, soluble starch, tartrate, erythritol and mannitol; L-sorbose, citrate and adonitol were not utilized. The carbohydrates selected for mutant screening experiments (Table 1) were chosen as effective but inexpensive carbon sources. Galactose, lactose and sorbitol are utilized after a period of adaptation.

Mutants isolated

The results of a number of mutant screening experiments are summarized in Table 1. The initial plates bore between 110 and 170 colonies each, and auxotrophic mutants (unable to grow on minimal medium but growing on complete medium) were isolated from them at a rate of 5·35 auxotrophs/1000 colonies. Mutants which did not grow on galactose occurred most frequently, but a few mutants were

Table 1. *Mutants in* Aspergillus nidulans *failing to utilize specific carbohydrates for growth*

Strain irradiated and no. of experiments	Total colonies	Auxotrophic mutants	Sugar mutants						Designation of the mutants
			*Fructose	Galactose	Lactose	Maltose	Sorbitol	Sucrose	
y2; *pyro4*									
3	3,371	21	2	†	1	1	0	0	*fr1*; *fr2*; *lac1*; *mal1*
bi1; *w3*									
3	9,839	40	1	8	6	1	7	0	*fr3*; *gal1-8*; *lac2-7*; *mal2*; *sb3-9*
10	16,517	97	†	28	†	†	†	†	*gal9-36*
	29,727	158	3	36	7	2	7	0	

The mutants were identified by replica plating followng u.v.-irradiation (survival rates 4·4–10·7 %) by their failure to grow on a given sugar as sole carbon source.

* The mutants were isolated by their failure to grow on either sorbitol or sucrose.

† Not tested.

obtained for each of the other sugars tested except sucrose. The mutants were tested for their ability to grow on a variety of sugars as sole carbon source; it was generally found that they were unable to utilize one particular sugar. Three exceptions, involving fructose, maltose and some of the galactose mutants, are described below. Mutants of the type described in *Escherichia coli* which fail to grow on glucose but grow on a number of other sugars, including maltose (Doudoroff *et al.* 1949), would have been detectable in these experiments, but none was found.

Location and characteristics of the mutants

The results of the analyses of the mutants are summarized in Table 2 which describes the loci identified, and Fig. 1 in which the position of loci in the genetic maps of the organism are shown. The mutants showed one of three characteristic phenotypes when tested for growth on the relevant sugars. In one the conidia germinated but did not grow beyond this stage; these are described as 'total mutants'. In a second phenotype the conidia germinated and formed very sparse colonies which produced a few conidial heads, the colonies increased in diameter at about the same rate as the wild type, but the density of the mycelium was greatly decreased. This 'starvation' growth is characteristic of *Aspergillus nidulans* when growth is limited by certain nutrients (Professor G. Pontecorvo, personal communication). Mutants of this second sort are described as 'partial mutants'.

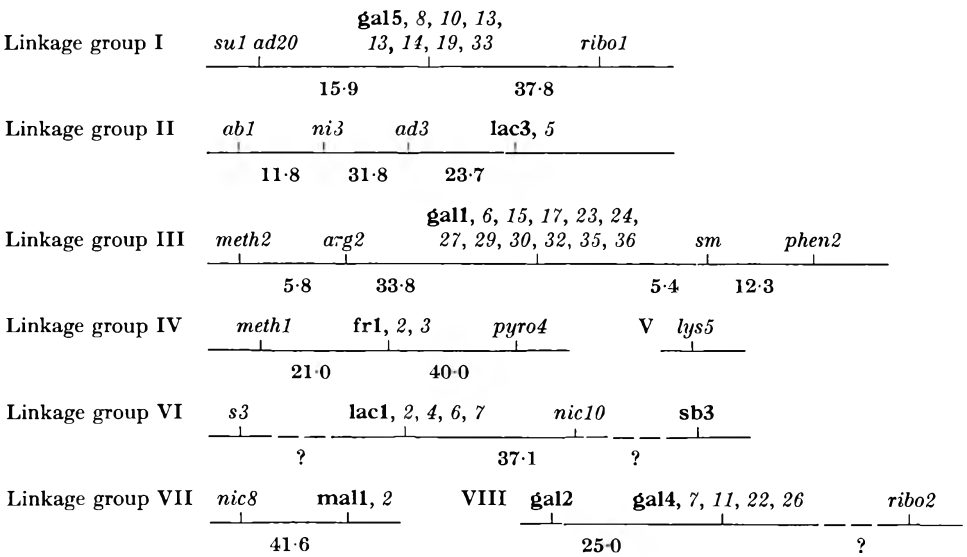

Fig. 1. Location of new markers (heavy type) in *Aspergillus nidulans*. Distances in meiotic recombination percentage. Symbols of mutant alleles: requirements, *ribo1*, *ribo2*, = riboflavin; *ab1* = γ-aminobutyric acid; *ni3* = nitrite; *ad3* = adenine; *meth1*, *meth2* = methionine; *arg2* = arginine; *phen2* = phenylalanine; *pyro4* = pyridoxin; *lys5* = lysine; *s3* = sulphite; *nic8*, *nic10* = nicotinic acid. *su1 ad20* = suppressor of *ad20*; *sm* = small colony. (See Pontecorvo *et al.* 1953; Käfer, 1958, for further details.)

In mutants of a third phenotype the conidia germinated and produced morphologically abnormal colonies which grew slowly as compared to the wild type and formed few or no conidial heads. Only a few mutants of this type, called 'slow growing mutants', were isolated and these were all galactose mutants. Among the mutants isolated, those at a particular locus all tended to have the same phenotype (Table 2). The mutant characters usually segregated 1:1 from the wild type both at meiosis and in mitotic haploidization. They are therefore determined by differences at single loci. The mutants are all recessive to wild type both in diploids and in heterokaryons.

Table 2. *Loci controlling carbohydrate utilization identified in* Aspergillus nidulans

Locus*	Phenotype†	Linkage group	No. of mutants	Allelic mutants
	Utilization of:			
gal1	Galactose (T)	III	12	*gal6, 15, 17, 23, 24, 27, 29, 30, 32, 35, 36*
gal2	Galactose (S)	VIII	1	—
gal3	Galactose (S)	II	1	—
gal4	Galactose (S)	VIII	5	*gal7, 11, 22, 26*
gal5	Galactose (T)	I	7	*gal8, 10, 13, 14, 19, 33*
lac1	Lactose (P)	VI	5	*lac2, 4, 6, 7*
lac3	Lactose (P)	II	2	*lac5*
mal1	Maltose (P)	VII	2	*mal2*
sb3	Sorbitol (P, T)	VI	7	See text
fr1	Fructose (T)	IV	3	*fr2, 3*

* The symbols of the loci represent the sugars which the mutants fail to utilize.
† T = total mutant, P = partial mutant, S = slow-growing mutant (see text).
Ten galactose mutants (*gal9, 12, 16, 18, 20, 21, 25, 28, 31, 34*) recombine freely with *gal1*, *gal4* and *gal5*; they have not been tested in crosses with *gal2* or *gal3*.

Table 3. *Growth of galactose mutants in* Aspergillus nidulans *on galactose and lactose*

	Carbon source	
Mutant	Galactose	Lactose
gal1; *gal6*	No growth	Wild type
gal2; *gal3* *gal4*; *gal7*	Slow growing	Wild type
gal5; *gal8*	No growth	Partial growth

Table 4. *Complementarity tests of eight galactose mutants in diploids heterozygous for pairs of* gal *mutants in* trans *arrangement*

	gal2	*gal3*	*gal4*	*gal5*	*gal6*	*gal7*	*gal8*
gal1	+	+	+	+	−	+	+
gal2	.	+	+	+	(+)	+	+
gal3	.	.	+	+	(+)	+	+
gal4	.	.	.	+	(+)	(+)	+
gal5	.	.	.	.	(+)	+	−
gal6	.	.	.	.	.	(+)	(+)
gal7	.	.	.	.	.	.	+

\+ = Wild-type growth: complementary mutants.
(+) = Partial growth: partially complementing mutants.
− = No growth: non-complementary mutants.

Groups of mutants between which there is complementation

(*gal1, 6*) (*gal2*) (*gal3*) (*gal4*) (*gal5, 8*) (*gal7*)

Galactose mutants

Eight galactose mutants isolated in a first series of u.v.-irradiation experiments may be separated into three phenotypic groups according to their growth on galactose or lactose as shown in Table 3. One possible explanation of the poor growth of

gal 5 and *gal8* on lactose, though by no means the only one, is an inhibition of growth by galactose similar to that described in strains of *Escherichia coli* lacking phosphogalactose transuridylase and accumulating Gal-1-PO_4 (Yarmolinsky, Weismeyer, Kalckar & Jordan, 1959).

Complementarity tests were done by synthesizing heterozygous diploids between the mutants taken in all possible pairwise combinations, using strains of the type *paba1 y ad20*; gal_x and *bi1*; *w3*; gal_y. The results of the tests are shown in Table 4.

Only two pairs of mutants (*gal1* and *gal6*; *gal5* and *gal8*) are completely non-complementary, one pair (*gal4* and *gal7*) is partially complementary, and *gal6* is partially complementary in all combinations. The significance of the last result is not clear but the first two permit the mutants to be arranged in six complementing groups.

In addition, certain pairs of mutants were crossed using strains of the type *paba1 y ad20*; gal_x and *bi1*; *w3*; gal_y. From each cross a suspension of ascospores from a single hybrid perithecium was plated on complete medium in dilutions yielding about 25 colonies per dish. Approximately 100 colonies from each cross were tested individually for their growth on galactose.

No recombinants were detected among progeny derived from the cross between the two partially complementing mutants (*gal4* and *gal7*) which are assumed to represent a single locus. The remaining groups of complementing mutants recombine at readily detectable frequencies and therefore the eight galactose mutants define five loci controlling galactose utilization. In one case (*gal7* × *gal2*) the segregation ratio (91 mutant:13 wild) was significantly greater than 3 mutant:1 wild, indicating linkage between *gal2* and *gal7*.

In later u.v.-irradiation experiments (see Table 1) a further 28 galactose mutants were isolated and these have been tested semi-quantitatively for linkage to *gal1*, *gal4* or *gal5* by means of a standard technique used in this laboratory. Crosses were set up between the new mutants and the three located mutants, hybrid perithecia selected and samples of ascospores streaked on galactose medium. The resulting growth was clearly either mutant or wild type and as each sample contained of the order of 200 ascospores the test could detect recombination down to about 1 % between the known galactose mutants and the new mutants. At this level of analysis 10 of the 28 new mutants were found not to recombine with *gal1*, 3 with *gal4* and 5 with *gal5* (Table 2). The remaining 10 new mutants which recombined freely with *gal1*, *gal4* and *gal5* have not been tested in crosses with *gal2* or *gal3*.

The gal1 *locus*. The segregation of markers in haploid strains derived by mitotic haploidization from the diploid between a tester strain (Forbes, 1959) and *bi1*; *w3*; *gal1* showed that *gal1* is located in linkage group III. This result was confirmed by meiotic analysis (Table 5): *gal1* is between *arg2* and *sm*, and shows about 34 % recombination with the former and about 5 % with the latter.

The gal2 *and* gal4 *loci*. Mitotic haploidization, using a tester strain, suggests that *gal4* is located in group VIII. However, the number of haploids tested is small and the result requires confirmation. Haploidization shows that *gal2*, which is linked to *gal4*, is not located in groups I, II, III, IV, V or VII, but no tests have been made with a tester strain marked in groups VI or VIII. Neither *gal2* nor *gal4* has been tested for meiotic recombination with group VIII markers, and location of these markers in group VIII is provisional.

The gal3 *locus.* Mitotic haploidization located *gal3* in linkage group II. Crosses involving *gal3* and *w3*, also in linkage group II, showed no linkage between these loci.

The gal5 *locus.* Mitotic haploidization located *gal5* in linkage group I. This is confirmed by meiotic analysis (Table 6), and the locus is between *ribo1* and *su1 ad20*, showing about 38 % recombination with the former and about 16 % with the latter.

Table 5. *Location of* gal1 *in linkage group III by meiotic analysis*

Cross: *meth2 arg2* + + + / + + *gal1 sm phen2*

Interval: (1) (2) (3) (4)

Genotype of progeny					No.	Class
meth	*arg*	+	+	+	77	Parental
+	+	*gal*	*sm*	*phen*	60	
+	*arg*	+	+	+	4	Single cross-overs in (1)
meth	+	*gal*	*sm*	*phen*	4	
+	+	+	+	+	54	Single cross-overs in (2)
meth	*arg*	*gal*	*sm*	*phen*	17	
+	+	*gal*	+	+	2	Single cross-overs in (3)
meth	*arg*	+	*sm*	*phen*	4	
+	+	*gal*	*sm*	+	8	Single cross-overs in (4)
meth	*arg*	+	+	*phen*	9	
meth	+	+	+	+	3	Double cross-overs in (1) and (2)
+	*arg*	*gal*	*sm*	*phen*	0	
meth	+	*gal*	+	+	0	Double cross-overs in (1) and (3)
+	*arg*	+	*sm*	*phen*	1	
meth	+	*gal*	*sm*	+	0	Double cross-overs in (1) and (4)
+	*arg*	+	+	*phen*	1	
meth	*arg*	*gal*	+	+	1	Double cross-overs in (2) and (3)
+	+	+	*sm*	*phen*	1	
meth	*arg*	*gal*	*sm*	+	2	Double cross-overs in (2) and (4)
+	+	+	+	*phen*	5	
meth	*arg*	+	*sm*	+	0	Double cross-overs in (3) and (4)
+	+	*gal*	+	*phen*	2	
+	*arg*	*gal*	+	+	0	Triple cross-overs in (1), (2) and (3)
meth	+	+	*sm*	*phen*	0	
+	*arg*	*gal*	*sm*	+	1	Triple cross-overs in (1), (2) and (4)
meth	+	+	+	*phen*	1	
+	*arg*	+	*sm*	+	0	Triple cross-overs in (1), (3) and (4)
meth	+	*gal*	+	*phen*	0	
+	+	+	*sm*	+	1	Triple cross-overs in (2), (3) and (4)
meth	*arg*	*gal*	+	*phen*	2	
meth	+	+	*sm*	+	0	Multiple cross-overs in (1), (2), (3) and (4)
+	*arg*	*gal*	+	*phen*	0	
				Total	260	

Recombination fractions (%):

gal1–meth2	$39{\cdot}5 \pm 3{\cdot}0$	*meth2–arg2*	$5{\cdot}8 \pm 1{\cdot}7$
gal1–arg2	$33{\cdot}8 \pm 2{\cdot}9$	*arg2–sm*	$35{\cdot}4 \pm 2{\cdot}9$
gal1–sm	$5{\cdot}4 \pm 1{\cdot}6$	*sm–phen2*	$12{\cdot}3 \pm 2{\cdot}0$
gal1–phen2	$15{\cdot}8 \pm 2{\cdot}3$		

Table 6. *Location of* gal5 *in linkage group 1 by meiotic analysis*

Cross:	*su1ad20*	*gal5*	*ribo1*
	+	+	+
Interval:		(1)	(2)

Genotype of progeny			No.	Class
su ad	*gal*	*ribo*	55	Parental
+	+	+	52	
+	*gal*	*ribo*	1	Single cross-overs in (1)
su ad	+	+	17	
+	+	*ribo*	24	Single cross-overs in (2)
su ad	*gal*	+	38	
su ad	+	*ribo*	7	Double cross-overs in (1) and (2)
+	*gal*	+	7	
		Total	201	

Recombination fractions (%)

su1ad20–gal5	15·9 ± 2·6
gal5ribo1	37·8 ± 3·4
su1ad20–ribo1	46·7 ± 3·5

Table 7. *Location of* lac1 *in linkage group VI by mitotic haploidization*

Classification of haploid strains isolated from the diploid

Tester *A/bi1*; *w3*; *lac1*

		phen2				+				
		pyro4		+		*pyro4*		+		
		lys5	+	*lys5*	+	*lys5*	+	*lys5*	+	
nic8	*lac1*	0	0	2	2	1	0	0	2	7
	+	0	0	2	1	1	0	0	1	5
+	*lac1*	3	1	2	3	0	0	0	0	9
	+	1	4	0	3	0	0	1	1	10
		4	5	6	9	2	0	1	4	

Tester *C/bi1*; *w3*; *lac1*

		ribo2	+	
s3	*lac1*	0	0	0
	+	3	6	9
+	*lac1*	8	11	19
	+	0	0	0
		11	17	

Tester strains contain markers identifying linkage groups. Tester A has markers in groups III (*phen2*), IV (*pyro4*), V (*lys5*) and VII (*nic8*); Tester C in groups VI (*s3*) and VIII (*ribo2*). A new marker is allocated to a linkage group by its failure to recombine with the marker defining the linkage group in haploid strains arising by mitotic haploidization from the diploid synthesized between a tester strain and a strain carrying the new marker. In this case *lac1* recombines freely with all the markers tested except *s3*. Absence of recombinants of the type *s3 lac1* or $s3^{+}$ $lac1^{+}$ is expected if *s3* and *lac1* are linked and in repulsion in the original diploid. This technique is fully described by Pontecorvo & Käfer (1958) and by Forbes (1959).

Lactose mutants

Seven phenotypically similar partial mutants were isolated and these classified into two complementing groups, which identify two loci, called *lac1* and *lac3*, controlling the utilization of lactose (Table 2).

The lac1 *locus.* Mitotic haploidization located *lac1* in linkage group VI which is defined by the marker *s3* (Table 7). In a cross including four group VI markers weak linkage was detected (37 % recombination) between *lac1* and *nic10*, but not between either of these markers and *s3* or *sb3* (Table 8).

Table 8. *Location of* lac1 *in linkage group VI by meiotic analysis*

Cross: $\frac{+\quad +\quad lac1\quad sb3}{s3\quad nic10\quad +\quad +}$

Interval: (1) (2) (3)

Genotype of progeny				No.	Class
+	+	*lac*	*sb*	28	Parental
s	*nic*	+	+	23	
s	+	*lac*	*sb*	19	Single cross-overs in (1)
+	*nic*	+	+	22	
s	*nic*	*lac*	*sb*	9	Single cross-overs in (2)
+	+	+	+	13	
s	*nic*	+	*sb*	21	Single cross-overs in (3)
+	+	*lac*	+	25	
+	*nic*	*lac*	*sb*	15	Double cross-overs in (1) and (2)
s	+	+	+	13	
+	*nic*	+	*sb*	26	Double cross-overs in (1) and (3)
s	+	*lac*	+	22	
+	+	+	*sb*	18	Double cross-overs in (2) and (3)
s	*nic*	*lac*	+	15	
s	+	+	*sb*	15	Triple cross-overs
+	*nic*	*lac*	+	12	
		Total		296	

Recombination fractions (%):

s3–nic10	48·0	*nic10–lac1*	37·1 ± 2·8
s3–lac1	48·0	*nic10–sb3*	48·0
s3–sb3	46·0	*lac1–sb3*	51·0

The lac3 *locus.* The mutant, *lac5*, which is non-complementary to *lac3*, was used in mapping this locus. Mitotic haploidization located *lac5* in linkage group II. Three crosses involving altogether 8 group II markers, including the nutritional markers *ribo6* and *ab1*, located by other workers in group II but not mapped, were analysed in attempting to locate *lac5*. The two nutritional markers were located successfully and no linkage was detected between *lac5* and the following markers: *ad23*, *Acr1*, *w3*, *ribo6*, *pu1*, *ab1* or *ni3* (Fig. 2). In one cross weak linkage was detected between *ad3* and *lac5* but both these markers showed very poor viability. If it be assumed that the viability factors operate independently in the double mutant *ad3 lac5*, the recombination fraction can be estimated after calculating the values of the viability factors. (I am indebted to Dr J. S. Gale for suggestions about

these calculations.) In this way a value of 23·7 % was obtained for the recombination fraction between these two loci. It is desirable that location of *lac5* should be confirmed by analysis of further crosses particularly including the marker *acr2*.

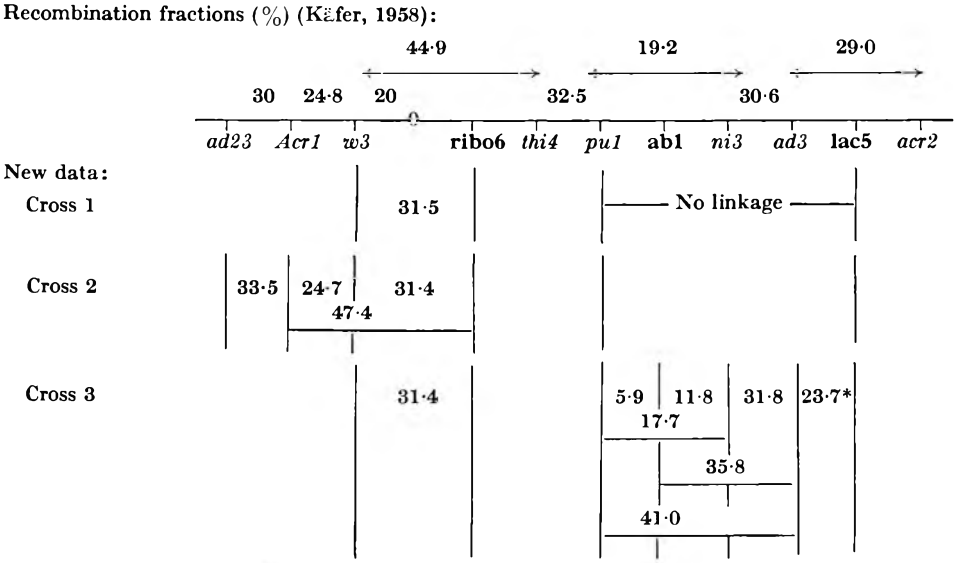

Fig. 2. Location of *lac5* in linkage group II by meiotic analysis. The vertical bars indicate markers present in the crosses. The newly mapped markers are in heavy type.

* This recombination fraction is calculated on the assumption that there is no viability interaction between *ad3* and *lac5* (see text). Symbols of mutant alleles: requirements, *ad23*, *ad3* = adenine; *ribo6* = riboflavin; *thi4* = thiamine; *pu1* = putrescine. Drug resistance, *Acr1* (dominant), *acr2* (recessive) = resistance to acriflavine; *w3* = white conidia. See also Fig. 1.

Table 9. *Location of* mal1 *in linkage group VII by mitotic haploidization*

Classification of haploid strains isolated from the diploid

Tester *A/bi1*; *w3*; *mal1*

		phen2				+				
		pyro4		+		*pyro4*		+		
		lys5	+	*lys5*	+	*lys5*	+	*lys5*	+	
nic8	*mal1*	0	0	0	0	0	0	0	0	0
	+	3	1	4	3	3	3	1	3	21
+	*mal1*	5	3	3	6	6	1	5	3	32
	+	0	0	0	0	0	0	0	0	0
		8	4	7	9	9	4	6	6	

See Table 7 for explanation.

Maltose mutants

Both the maltose mutants isolated grow poorly on sucrose, which suggests that they are defective in α-glucosidase. The mutants are non-complementary and identify a locus located in linkage group VII by mitotic haploidization (Table 9).

Weak linkage (41·6 % recombination) was detected between *mal1* and *nic8* in a cross between these markers.

Fructose mutants

Three fructose mutants were isolated: one (*fr1*) was detected by failure to grow on sucrose and was originally given the symbol *suc1* (this symbol was used by Forbes, 1959), and the other two (*fr2* and *fr3*) by failure to grow on sorbitol and originally called *sb1* and *sb2*, respectively. All three mutants are identical phenotypically in that they fail to grow on either fructose, sorbitol or sucrose. The mutants are apparently inhibited by fructose or a metabolic product of fructose, for they fail to grow on glucose or other sugars to which small quantities of fructose have been added, and fail to utilize carbohydrates which either contain a fructose residue, such as sucrose, or are metabolized via fructose as in the case of sorbitol (Shockley & Pride, 1959).

The three mutants are non-complementary and define a single locus concerned with fructose utilization which was located in linkage group IV by means of haploidization of diploids and mapped meiotically between *meth1* and *pyro4* (Table 10).

Table 10. *Location of* fr1 *in linkage group IV by meiotic analysis*

Cross: $\frac{meth1 \quad + \quad pyro4}{+ \quad fr1 \quad +}$

Interval: (1) (2)

Genotype of progeny			No.	Class
meth	+	*pyro*	70	Parental
+	*fr*	+	77	
+	+	*pyro*	22	Single cross-overs in (1)
meth	*fr*	+	16	
+	*fr*	*pyro*	43	Single cross-overs in (2)
meth	+	+	54	
meth	*fr*	*pyro*	10	Double cross-overs in (1) and (2)
+	+	+	17	
		Total	309	

Recombination fractions (%):

meth1–fr1	21·0 ± 2·3
fr1–pyro4	40·0 ± 2·8
meth1–pyro4	43·5 ± 3·1

Sorbitol mutants

The sorbitol mutants are distinguishable from the fructose mutants by their ability to grow on sucrose and fructose. The seven mutants isolated are of two types; two are total mutants (*sb3*, *sb5*) and the remaining five, partial mutants (*sb4*, *sb6*, *sb7*, *sb8*, *sb9*). In the present state of analysis a number of ambiguities have not been resolved and the relationship of the mutants has not been established.

The mutants were combined in all possible pairs in heterozygous diploids synthesized between strains of the type *bi1*; *w3*; sb_x and *ad14 paba1 y*; sb_y. When the diploids are tested for their growth on sorbitol three pairs of mutants are non-complementary (*sb3* and *sb5*; *sb4* and *sb7*; *sb6* and *sb9*) but complement in all other

combinations, while *sb8* is non-complementary in all combinations. This result is described by the primitive complementation map shown in Fig. 3. Three of the mutants (*sb3*, *sb4*, *sb9*) were located in linkage group VI by mitotic haploidization (Table 11). Tests of *sb3* in crosses involving three other group VI markers (Table 8)—*s3*, *nic10* and *lac1*—showed free recombination at meiosis between the *sb* locus and all three others.

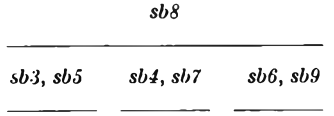

Fig. 3. Complementation of sorbitol mutants in heterozygous diploids of *Aspergillus nidulans*.

Table 11. *Location of* sb3, sb4 *and* sb6 *in linkage group VI by mitotic haploidization*

Classification of haploid strains isolated from the diploids

Tester *C/bi1; w3; sb3, sb4* and *sb9*

		ribo2	+					*ribo2*	+	
s3	*sb3*	0	0	0	*s3*	*sb4*	0	0	0	
	−	4	1	5		+	17	8	25	
+	*sb3*	4	2	6	+	*sb4*	12	9	21	
	+	0	0	0		+	0	0	0	
		8	3				29	17		

		ribo2	+	
s3	*sb9*	0	0	0
	+	1	3	4
+	*sb9*	7	5	12
	+	0	0	0
		8	8	

See Table 7 for explanation.

In testing the sorbital mutants for functional relationships the unusual result was observed that although certain pairs of mutants complement in heterozygous diploids, none of the pairs of mutants complements in heterokaryons. Balanced heterokaryons were synthesized between pairs of strains of the type *bi1*; *w3*; sb_x and *ad14 paba1 y*; sb_y. The heterokaryons were tested by transferring actively growing mycelium from minimal medium to a test medium of basal medium + sorbitol. They all showed partial growth, that is they were all mutant. Supplementing this test medium with nutrients required by the component strains, either altogether or in various combinations, did not appreciably improve the growth of the mycelium. Variation of the incubation temperature was also without effect. The heterokaryotic mycelia continued to show partial (mutant) growth for at least three serial subcultures on unsupplemented basal medium + sorbitol but always immediately yielded vigorously growing heterokaryons on return to minimal medium. That the difference between complementarity of pairs of sorbitol mutants in heterokaryons and in heterozygous diploids is a function of the *sb* mutants themselves, is shown by the fact that it appears also between other heterokaryons and diploids with the same combinations of *sb* mutants but with different combinations of nutritional markers, namely *ad14 paba1 y* and *ribo1 pro1 bi1*; *w3*.

DISCUSSION

This paper emphasizes the usefulness of the new type of mutants in mould genetics. In *Aspergillus nidulans* the mutants segregate clearly in both meiotic and mitotic recombination, they usually have good viability, and they can be used in selective plating techniques.

The possibility that differences in phenotype may occur between combinations of the same genes in heterokaryons and in heterozygotes was predicted by Pontecorvo (1952*b*) and has been discussed more recently by other authors (Roper, 1958; Lewis, 1961). Differences have been described in Ustilago in the pathogenicity and saprophytic growth of dikaryons as compared with diploids (Holliday, 1961) and also in the case of *pan2* mutants of Neurospora combined in heterokaryons or in disomic 'pseudowild' strains (Case & Giles, 1960). The series of sorbitol mutants described here, which are non-complementary in heterokaryons, although certain pairs complement in heterozygous diploids, constitutes a further example in *A. nidulans*. Two facts suggest that the difference is a property of the mutants rather than a nutritional interaction or the result of an unfavourable nuclear balance in the heterokaryons. First, the mutants do not complement in heterokaryons when the test medium is supplemented with mixtures of the nutrients required by the component strains, that is when provided with the opportunity for a shift in the nuclear ratio. Secondly, they also fail to complement when tested in combinations with different nutritional markers.

Recent work by the author has shown that the oxidation of sorbitol by intact organisms involves an inducible enzyme system; experiments to test for differences in enzyme formation between the heterokaryons and heterozygotes will be described in a later publication. It is clearly necessary to distinguish genetically between the possibilities that the sorbitol mutants represent three cistrons spanned by a deletion (*sb8*) or a single cistron within which complementation occurs between some alleles (Catcheside & Overton, 1958; Catcheside, 1960). In the first case models could be built (Professor G. Pontecorvo, personal communication) in which the mutants involve regulating and structural cistrons (Jacob & Monod, 1961) and failure of the mutants to complement across the cytoplasm results from differences in the local concentrations of the apo-repressor. If the mutants involve only structural cistrons, analogous to those which determine the structure of the A and B components of *Escherichia coli* tryptophan synthetase (Yanofsky, 1960), complementation may only occur in the heterozygotes as the result of interaction of gene products at a state intermediate between DNA and polypeptide—as suggested by Dorn & Burdick (1962) for an example in Drosophila—rather than an interaction of protein subunits in the cytoplasm as is commonly held (see Catcheside, 1960). Phenomena of the sort described here may well prove more common as studies of the genetics of moulds are extended, and may be of interest in the study of protein synthesis and the mechanisms of intra-cistron complementation.

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Isolation of Group Specific Products from *Lactobacillus casei* and *L. casei* var. *rhamnosus*

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SUMMARY

An examination has been made of serologically reactive products obtained from four strains of *Lactobacillus casei* (serological groups B and C) and two strains of *Lactobacillus casei* var. *rhamnosus* (serological group C). Cell wall preparations were hydrolysed with a preparation of Streptomyces muralytic enzyme and the soluble indiffusible products isolated. Rhamnose is the major component of both the cell wall and soluble extracts from group B organisms, whereas glucose is the major component of preparations from *L. casei* group C. Two products differing considerably in composition have been isolated from each strain of *L. casei* var. *rhamnosus*.

The serological properties of the preparations have also been studied and the results confirm that strains of *Lactobacillus casei* can be divided into two serological groups. It is concluded that group B specificity is primarily dependent on rhamnose and group C specificity on glucose, which is probably joined by a 1–6 β linkage to the adjacent sugar. The two fractions isolated from *L. casei* var. *rhamnosus* differ in their serological properties; antibodies formed on injection of this organism are predominantly directed against one component (in fraction I) whereas extracts react with sera against strains of *L. casei* group C because of the presence of a second component (in fraction II).

INTRODUCTION

In an extensive serological study of lactobacilli Sharpe (1955*a*) found that 70% of the strains tested could be classified into six groups and one subgroup. It was shown that a serological group may contain more than one species and further that organisms classified as *Lactobacillus casei* could be divided into two serological groups, one of which also contained organisms designated *L. helveticus*. Subsequently Sharpe & Wheater (1957) proposed that the species previously defined as *L. helveticus* be renamed *L. casei* var. *rhamnosus* and that the serological groups be referred to by letters, group B including *L. casei* and group C including *L. casei* and *L. casei* var. *rhamnosus*. Sharpe used hot dilute acid extracts of lactobacilli for her serological studies. However, to investigate the chemical basis of the serological specificity of *L. casei* a milder technique for obtaining soluble extracts was considered desirable and a previous report (Knox & Brandsen, 1962) described the isolation of serologically active soluble products from autolysed cell wall of a strain of *L. casei* (group B). This technique was found to have restricted application and the present report describes the properties of products released by *Streptomyces albus* enzymes from the cell wall of Lactobacillus of serological groups B and C. This

streptomyces enzyme system was shown by McCarty (1952*b*, 1956, 1958) to dissolve the cell wall of Streptococcus of Lancefield group A, the soluble products including the group-specific carbohydrate hapten.

METHODS

Organisms. Cultures of Lactobacillus used by Sharpe (1955*a*) were obtained from the National Collection of Type Cultures, Colindale, London (designated NCTC), the National Institute for Research in Dairying, Reading, England (NIRD) and the National Collection of Industrial Bacteria, Torry Research Station, Aberdeen, Scotland (NCIB). These organisms were *Lactobacillus casei* NIRD strain H 831 and *L. casei* NIRD DECP belonging to group B, and *L. casei* NIRD R 094 and *L. helveticus* NCTC 6375 of group C. A strain of *L. delbrueckii* (NCIB 7473) has also been included, since the wall of this strain was shown by Cummins & Harris (1956) to contain rhamnose, a characteristic component of the cell wall of *L. casei*. However, on the basis of physiological and serological tests this strain is considered to be *L. casei* var. *rhamnosus* (group C). The laboratory strain 55 of *L. casei* (group B) used in previous studies (Knox & Brandsen, 1962) was also included in the current investigation.

Culture media. Cultures for inoculation into rabbits and for the preparation of HCl extracts were grown at 37° in the appropriate media described by Sharpe (1955*b*). Washed organisms were suspended in 0·4% formaldehyde + 0·9% NaCl solution and injected intramuscularly in rabbits every 3–4 days for a 4-week period. Organisms for disintegration and isolation of cell wall were grown in the medium described by Agren & de Verdier (1958).

Preparation of cell wall. Suspensions of organisms (equivalent to about 25 mg. dry wt./ml.) were shaken with Ballotini No. 12 glass beads in a Mickle disintegrator for 1 hr. (Salton & Horne, 1951). The cell-wall residue was suspended in M/15 phosphate (pH 7·8) heated at 100° for 20 min. (to inactivate cell-wall autolysing enzymes) and then digested with crystalline trypsin and ribonuclease (Armour Laboratories, Eastbourne, England) as described by Cummins & Harris (1956). The wall preparation was subsequently washed repeatedly with M/15 phosphate buffer (pH 7·2) then with water and dried from the frozen state.

Analytical methods. Methods for the determination of nitrogen, glucose, rhamnose, hexosamines and amino acids were as described previously (Knox & Brandsen, 1962). The galactose content of preparations was calculated from the total hexose value, determined by the basic cysteine reaction (Dische, 1949). Galactose and rhamnose gave 90 and 5%, respectively, of the amount of colour given by an equal weight (200 μg.) of glucose. The reproducibility of the results for wall preparations was improved by hydrolysing samples for 2 hr. at 100° in N-H_2SO_4 before carrying out the estimations.

Preparation of the Streptomyces albus *muralytic enzyme.* Murphy (1960) suggested that 'muralytic enzyme' be used as a generic term for those enzymes which lyse bacteria by dissolving the cell wall. McCarty (1952*a*, *b*) showed that certain strains of *Streptomyces albus* possessed a muralytic enzyme acting on streptococci; cultures of *S. albus* with such activity were provided by Dr M. McCarty (Rockefeller Institute, New York, U.S.A.) and Dr J. A. Hayashi (College of Medicine, University of Illinois, Chicago, U.S.A.). Other strains of Streptomyces isolated from soil by the technique described by Salton (1955) were less effective.

The *Streptomyces albus* enzyme was prepared by the method described by McCarty (1952*a*, *b*). After precipitation by 70% saturation with $(NH_4)_2SO_4$ in the presence of Filter-Cel (Johns-Manville, Lompoc, California, U.S.A.), the enzyme was dissolved in M/15 phosphate buffer (pH 8); this solution was used for dissolving cell wall without further fractionation. As measured by the decrease in optical density (at 620 mμ) the enzyme was capable of dissolving the cell wall of each of the strains of Lactobacillus examined, although there were differences in the speed.

RESULTS

Properties of the streptomyces muralytic enzyme

A preparation of wall from Lactobacillus strain NCTC 6375 (40 mg. in 20 ml. water) was incubated at 37° with 4 ml. of dialysed enzyme (dialysis at 2° against distilled water did not affect the activity of the enzyme preparation). The rate of

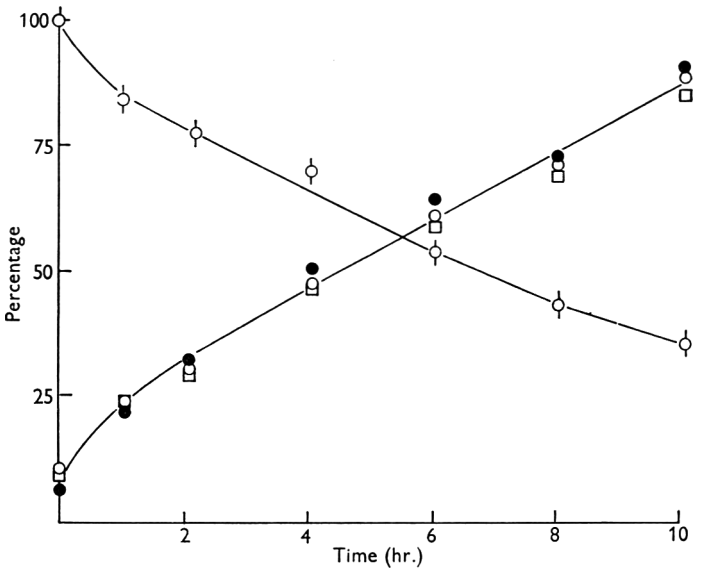

Fig. 1. The lysis of cell wall of Lactobacillus strain NCTC 6375 by streptomyces enzyme. The decrease in optical density (ϕ) is expressed as percentage of the starting value. The amount of rhamnose (□), glucose (○) and hexosamine (●) in the soluble fraction is expressed as percentage of the total amount in the sample of cell wall.

decrease in optical density at 620 mμ was followed (using ½ in. tubes in a Unicam SP 1400 Absorptiometer) and at appropriate intervals a 2 ml. sample was removed and added to 2 ml. N-H_2SO_4. The cell wall residue was removed by centrifugation, the supernatant fluid heated at 100° for 4 hr. to hydrolyse the polysaccharides, and the rhamnose, glucose and hexosamine contents determined. In Fig. 1 the rate of release of these components is compared with the rate of decrease in optical density.

The optimal pH value for enzyme activity was determined by following the rate of decrease in optical density in M/15 phosphate buffer at pH values between 5·3 and 8·0. (At pH values below 5·0 the wall flocculated.) The maximum rate of enzyme action was observed between pH 5·3 and 6·5, with decreasing activity at

higher values. For instance, in 2 hr. at pH 5·9 the optical density decreased by 36% as compared with 27% at pH 8·0; incubation for 3 hr. at pH 8 was required to decrease the optical density by 36%.

After incubation for 3 hr. the suspensions were heated to inactivate the muralytic enzyme (100° for 20 min.), centrifuged and the supernatant fluid dialysed. The rhamnose and glucose contents of the indiffusible fraction were then determined. The results supported the conclusion that maximum enzymic activity was obtained between pH 5·3 and 6·5. Again, comparing the results at pH 5·9 and 8·0, the amounts of soluble rhamnose and glucose at pH 8 represented 57 and 62%, respectively, of the values obtained at pH 5·9. At all pH values between 5·3 and 8·0 there was no significant change in the relative molar proportions of rhamnose and glucose present in the indiffusible fraction.

Most of the soluble polymers containing rhamnose and glucose were indiffusible. A preparation of wall from Lactobacillus strain NIRD H831 was suspended in water and incubated for 18 hr. at 37° with dialysed streptomyces enzyme. The cell wall residue was then removed by centrifugation and the supernatant fluid dialysed at 2°. Of the total soluble rhamnose, glucose and glucosamine, only 2–4% was present in diffusible polymers, whereas 27% of the total muramic acid was diffusible. No monosaccharides were detectable.

Analysis of cell wall preparations

As shown previously (Knox & Brandsen, 1962), the wall of Lactobacillus strain 55 contains rhamnose, galactose and glucose in addition to the characteristic components of the cell wall (glucosamine, muramic acid, glutamic acid, aspartic acid, lysine and alanine).

The cell wall preparations from each of the five additional strains also contain rhamnose, glucose, glucosamine, galactosamine and muramic acid. All except Lactobacillus strain NIRD DECP contain galactose and strain NCIB 7473 in addition contains mannose. The results of the quantitative analyses for these components are given in Table 1. The major difference in the preparations from the different strains lies in the relative molar proportions of rhamnose and glucose; the values for this ratio are given in Table 2.

The major amino acids of the cell wall of each strain were: glutamic acid, aspartic acid, lysine, alanine. In contrast to the wide range of rhamnose and glucose values there was a much greater constancy in the molar proportions of the amino acids present in the wall. The molar ratios of glutamic acid, aspartic acid, lysine and alanine for the six strains fell within the range 1·0:0·6–1·2:0·7–1·0:1·6–2·0.

Preparation of cell wall carbohydrates

In earlier experiments, the mixture of cell wall and muralytic enzyme was incubated at pH 8 as suggested by McCarty (1952*b*). When it was observed that a more rapid dissolution of cell wall occurred at or below pH 6·5 experiments were made at pH 5·3. To illustrate the procedure for obtaining soluble cell wall products the results of a typical experiment at pH 8 will be described. A preparation of cell wall from Lactobacillus NIRD H831 (900 mg. in 50 ml. M/15 phosphate buffer) was incubated at 37° with an equal volume of streptomyces enzyme preparation. To

follow the rate of cell wall lysis, a sample (0·3 ml.) was removed and diluted with 4·0 ml. M/15 phosphate buffer (pH 8·0). The optical density of this suspension fell from 0·62 to 0·28 in 18 hr. and to 0·26 in 24 hr. After 24 hr. the remainder of the suspension was centrifuged and $(NH_4)_2SO_4$ added to the supernatant fluid to 70%

Table 1. *Comparison of the composition of cell wall preparations and soluble products liberated by streptomyces enzyme*

Lactobacillus strain	Fraction	Rhamnose (%)	Glucose (%)	Galactose (%)	Hexosamine* (%)	Muramic acid (%)	Nitrogen (%)
L. casei NIRD H831	Wall	33	2·0	7	16·2	9·8	7·6
	I	31	4·2	—	15·0	6·5	6·4
	II	48	3·6	7	24·2	5·2	3·6
L. casei NIRD DECP	Wall	26	11·5	0	11·2	3·8	6·6
	I	18·5	6·1	—	9·6	3·8	8·5
	II	39	17	0	18·4	2·0	4·2
L. casei strain 55	Wall	28	11	14	16·0	8·2	4·8
	I	16	4·2	—	7·4	1·0	9·9
	II	41	13	16	18·8	2·2	2·3
L. casei NIRD R094	Wall	1·5	12·5	13	17·6	12·0	8·2
	I	3·0	11	—	8·6	2·8	9·1
	II	1·7	27·5	23	21·2	5·8	3·8
L. helveticus NCTC 6375	Wall	24	22·5	12	5·0	1·0	4·8
	I	46	33	18	1·0	0·0	1·9
	II	2·0	28	27	14·0	1·0	3·9
L. delbrueckii NCIB 7473	Wall	28	23	18†	6·8	4·0	4·5
	I	56	35	0‡	1	0·0	1·2
	II	9·1	34	24§	20·0	2·8	3·5

* Glucosamine + galactosamine; † includes mannose; ‡ contains 9% mannose (determined by basic cysteine reaction); § includes trace of mannose.

Table 2. *Molar ratios of rhamnose and glucose in cell wall preparations and isolated soluble products of certain strains of Lactobacillus*

Serological group	Lactobacillus strain	Molar ratio of rhamnose to glucose		
		Wall	Fraction I	Fraction II
B	NIRD H831	18·4	8·6	14·8
	NIRD DECP	2·5	3·4	2·6
	strain 55	2·8	4·4	3·5
C	NIRD R094	0·13	0·30	0·09
	NCTC 6375	1·19	1·54	0·08
	NCIB 7473	1·34	1·77	0·30

saturation. At this level, the enzyme and possibly the cell wall components would be precipitated. The precipitate was dissolved in distilled water, and both the precipitate (fraction I) and the supernatant fluid (fraction II) from the $(NH_4)_2SO_4$ fractionation dialysed and the non-diffusible portions dried from the frozen state. The yields of cell wall residue, fraction I and fraction II, were 120 mg., 60 mg. and 550 mg. respectively. Fraction II was also the major product obtained from strains NIRD DECP, 55 and NIRD R094; for the other two strains (NCTC 6375 and

NCIB 7473) fraction I was the major component. For each of the six strains under examination the composition of fractions I and II has been compared with that of the wall (Table 1).

Analysis of cell wall carbohydrates

Fraction I. For four strains (Lactobacillus NIRD R094 and the three strains of group B) the fraction precipitated by 70% $(NH_4)_2SO_4$ represented only a small amount of the total soluble material; the analyses (Table 1) indicated that the products contained all the components of the cell wall. Enzyme protein would be contributing to the total nitrogen, which in two instances was considerably higher than the nitrogen content of the wall.

The preparations of fraction I from Lactobacillus NCTC 6375 and NCIB 7473 differed in composition from those obtained from the other strains, the nitrogen and hexosamine values being low and muramic acid absent. Rhamnose and glucose were the major components in each case, the remaining sugar being either galactose (strain NCTC 6375) or mannose (strain NCIB 7473). The nitrogen content of fraction I from these two strains could be lowered by further fractionation. The preparation was dissolved in ethylene glycol (1% w/v) and acetone added; 50% (v/v) acetone precipitated a small amount of material, the bulk of the material (fraction I*b*) being precipitated by 55% (v/v) acetone. For instance, fraction I*b* from strain NCTC 6375 contained 51% rhamnose, 37% glucose, 20% galactose and 0·4% nitrogen (1·9% nitrogen in fraction I).

Fraction II. Preparations of fraction II contained all the cell wall components including the amino acids, glutamic acid, aspartic acid, lysine and alanine; quantitative analyses for carbohydrates and nitrogen are given in Table 1. Preparations from Lactobacillus strains in serological group B had rhamnose : glucose ratios comparable to those of the cell wall preparations. Preparations from strains in serological group C were similar in composition with low rhamnose:glucose ratios (Table 2).

Three observations on the results in Tables 1 and 2 are relevant to the subsequent serological studies. First, organisms in serological group B have a higher rhamnose: glucose ratio than those in group C; this applies both to the cell wall preparations and to the soluble fractions. Secondly, preparations of fraction II from Lactobacillus strains of serological group C have a low rhamnose:glucose ratio. Thirdly, for each of the strains of *Lactobacillus casei* var. *rhamnosus* in serological group C (i.e. strains NCTC 6375 and NCIB 7473) fractions I and II differ considerably in composition—fraction I being composed almost entirely of rhamnose and glucose and fraction II containing much less rhamnose but the carbohydrate components of the mucopeptide.

Further fractionation of fraction II

In previous work (Knox & Brandsen, 1962) it was shown that the soluble material obtained on autolysis of cell wall from Lactobacillus strain 55 could be fractionated into products of different composition by dissolving in ethyleneglycol and precipitating with increasing amounts of acetone; the major difference between the fractions was in the relative amounts of rhamnose and glucose, the molar ratios varying between 0·9:1 and 5·0:1. In the present work it was found that fraction II from strain 55 also yields products of differing composition. From 100 mg. of

material was obtained (*a*) 28 mg. fraction II*a*, precipitated by 50% (v/v) acetone, and containing 50% rhamnose and 12% glucose (ratio 4·6:1); (*b*) 40 mg. fraction II*b*, precipitated by 60% (v/v) acetone, and containing 50% rhamnose and 10·5% glucose (ratio 5·3:1); and (*c*) 18 mg. fraction II*c*, precipitated by 65% (v/v) acetone+1% (w/v) NaCl, and containing 19% rhamnose and 16·5% glucose (ratio 1·3:1). Fraction II from strain NIRD DECP was also fractionated in a similar manner but a separation into products of different rhamnose:glucose ratios was not achieved.

Serological examination of soluble cell wall products

Sharpe (1955*a*) studied the antigenic relationship between strains of *Lactobacillus casei* and *L. helveticus* by the qualitative precipitin test. Sera prepared against *L. casei* NIRD H831 and *L. casei* NIRD DECP were used for classifying organisms in the *L. casei* group (subsequently called group B) and sera prepared against *L. casei* NIRD R094 and *L. helveticus* NCTC 6375 for classification in the *L. casei-helveticus* group (group C). *L. casei* strain 55 and *L. delbrueckii* NCIB 7473 were examined by this procedure and, as stated previously, it is concluded that these two strains belong to serological groups B and C respectively. The division of these organisms into two groups has been confirmed by studying the serological properties of the products liberated by the Streptomyces enzyme.

Table 3. *Cross reaction between antisera to three strains and the soluble products (fraction II) isolated from cell wall preparations of certain strains of Lactobacillus*

In each case 10 μg. of preparation was mixed with 0·1 ml. of serum in a final volume of 1·0 ml. The values are expressed as % of amount of precipitate obtained in the homologous reaction. When results are not listed the values were not significantly greater than those given by the blank containing serum and saline.

Lactobacillus source of Fraction II	Antiserum		
	H831	DECP	R094
	%		
NIRD H831	100	73	—
NIRD DECP	70	100	—
Strain 55	110	90	—
NIRD R094	—	—	100
NCTC 6375	—	—	84
NCIB 7473	—	—	98

Fraction I. The preparation of fraction I from Lactobacillus NCTC 6375 and NCIB 7473 reacted strongly with antisera to strain NCTC 6375 and also to strain NCIB 7473 but gave no detectable precipitate with antiserum to strain NIRD R094. An indication of the extent of cross reaction with antiserum to strain NCTC 6375 was obtained by the quantitative precipitin test. Solutions of fraction I from each strain were diluted to 20 μg./ml. in 0·9% NaCl and 0·5 ml. mixed with 0·5 ml. serum which had been diluted fivefold. Experiments were set up in triplicate. After 1 hr. at 37° and 2–3 days at 4° the protein content of the precipitate was determined by the method of Heidelberger & MacPherson (1943) by using Folin & Ciocalteu's phenol reagent. It was found that the preparation from strain NCIB 7473 gave 98% of the amount of precipitate given by NCTC 6375. A comparison of

the two preparations by the Ouchterlony method (Ouchterlony, 1953) showed the presence of a single serologically identical component.

Fraction II. Preparations were tested against each of the four group sera by the qualitative precipitin test. The results agreed with those obtained for acid extracts, although only weak cross reactions were obtained between antisera to Lactobacillus NCTC 6375 and the organisms of group C. An indication of the extent of cross reaction between the six strains was obtained by the quantitative precipitin test. Experiments were set up in triplicate against antisera to strains NIRD H831, NIRD DECP and NIRD R094, using the same conditions as described above for fraction I. In Table 3 the amount of precipitin obtained is expressed as a percentage of that given in the homologous reaction.

The observations were extended to include a study by the Ouchterlony technique (Ouchterlony, 1953), each of the six preparations of fraction II being tested against antisera to Lactobacillus NIRD H831 (serological group B) and NIRD R094 (serological group C). The test revealed only one component; within each serological group the fractions were shown to be serologically identical and no cross reaction between the groups was detectable.

Inhibition of precipitin reaction by component sugars

The effectiveness of 25 μmoles of rhamnose, galactose and glucose in inhibiting the precipitin reaction between antisera and fractions I and II was studied (Table 4). The amounts of antiserum and polysaccharide used were found suitable by preliminary studies in which the proportions of antiserum and polysaccharide were varied. The antiserum (0·2 ml. for group B organisms and 0·1 ml. for group C organisms) was mixed with 25 μmoles of rhamnose or glucose in a final volume of 0·5 ml. After 1 hr. at 37°, 0·5 ml. of solution containing the appropriate amount of polysaccharide was added and the amount of precipitate formed on standing was estimated as described previously.

Table 4. *Inhibition of precipitin reaction between antisera and preparations of fractions I and II by 25 μmoles of rhamnose, glucose or galactose*

Serological group	Lactobacillus strain	Amount of component added		Inhibition (%)		
		Fraction	Serum	Rhamnose	Glucose	Galactose
B	NIRD H831	20 μg. II	0·2 ml. H831	55	0	4
	NIRD DECP	10 μg. II	0·2 ml. DECP	60	7	—
	Strain 55	25 μg. II	0·2 ml. 55	47	14	25
		25 μg. II*b*	0·2 ml. 55	50	14	—
		25 μg. II*c*	0·2 ml. 55	30	27	—
C	NIRD R094	5 μg. II	0·1 ml. R094	11	25	3
	NCTC 6375	10 μg. I	0·1 ml. 6375	10	24	4
		10 μg. II	0·1 ml. R 094	0	26	2
	NCIB 7473	10 μg. I	0·1 ml. 6375	7	19	0
		10 μg. II	0·1 ml. R094	4	34	7

Fraction I. For examining preparations of fraction I from Lactobacillus NCTC 6375 and NCIB 7473, antiserum to strain NCTC 6375 gave satisfactory results. Although fraction I from strain NCIB 7473 reacted strongly with the homologous antiserum

it was not possible to obtain more than 4% inhibition of the precipitin reaction, even with 100 μmoles of rhamnose or glucose.

Fraction II. All preparations of fraction II from organisms of group B were tested using the homologous antiserum. Antiserum to Lactobacillus NIRD R094 was used for studies on fraction II from group C organisms. The study of strain 55 was extended to include fractions II*b* and II*c*, the products separated by acetone precipitation from ethyleneglycol in which the rhamnose:glucose ratios were 5·1:1 and 1·3:1 respectively.

Further studies on the glucose specificity of group C organisms

The ability of a number of glucosides to inhibit the precipitin reactions between antisera to organisms of group C and corresponding fractions has been studied, using the same conditions as those described above. The sugars employed were methyl-α-D-glucopyranoside, methyl-β-D-glucopyranoside and all the reducing disaccharides containing glucose units joined by β linkages—sophorose ($1 \rightarrow 2$), laminaribiose ($1 \rightarrow 3$), cellobiose ($1 \rightarrow 4$) and gentiobiose ($1 \rightarrow 6$).

Fraction I. The inhibition of the reaction of fraction I from Lactobacillus NCTC 6375 with homologous antiserum was studied. Methyl-β-D-glucoside was more effective than methyl-α-D-glucoside, the respective values for 25 μmoles being 28 and 20% compared with 24% for glucose. None of the disaccharides was more effective than methyl-β-D-glucoside.

Table 5. *Effect of periodate oxidation for 8 min. at room temperature on the component sugars and serological activity of fractions I and II*

Lactobacillus strain	Fraction	Amount of component remaining (expressed as % of starting value)		Serological reactivity (amount of precipitate expressed as % of starting value)
		Rhamnose	Glucose	
NIRD H831	II	54	—	26
NIRD R094	II	—	81	62
NCIB 7473	II	100	78	48
NCIB 7473	I	109	84	69
NCTC 6375	I	108	83	74

Fraction II. Methyl-β-D-glucoside was also more effective than methyl-α-D-glucoside in inhibiting the reaction between fraction II from Lactobacillus NIRD R094 and homologous antiserum, the inhibition by 25 μmoles being 42% compared with 12% (25% by glucose). The extent of inhibition by 10 μmoles of disaccharide has been studied; gentiobiose gave 33% inhibition, whereas the remaining disaccharides, as well as methyl-β-D-glucoside (10 μmoles) gave 21–24% inhibition. With smaller amounts (4 μmoles) of carbohydrate the corresponding values for gentiobiose and methyl-β-D-glucoside were 24 and 14% respectively.

Action of periodate on group specific products

Under the same conditions as those adopted by Foster, Davies & Crumpton (1958) preparations were oxidized for 3 and 8 min. and subsequently reduced by sodium borohydride. Samples were then analysed for component sugars, and for serological activity by the quantitative precipitin test; the amounts of components used in the precipitin test were the same as those given in Table 4. The results of the effect of periodate for 8 min. are summarized in Table 5.

Serological group B. Rhamnose is responsible for the serological specificity of Lactobacillus strains of group B, and for a strain in this group (NIRD H831) it was found that almost half the rhamnose was oxidized in 8 min. This result indicates that the rhamnose units are joined by either a 1 → 2 or a 1 → 4 linkage.

Serological group C. Glucose is responsible for the serological specificity of both products which have been isolated from group C organisms. The glucose units in fraction II from Lactobacillus NIRD R094 and NCIB 7473 were oxidized by periodate, oxidation being accompanied by reduction in serological reactivity. In the absence of more complete analyses, it is only possible to state that glucose units responsible for serological specificity are joined by 1 → 2, 1 → 4 or 1 → 6 linkages. The glucose units in fraction I from strain NCIB 7473 and NCTC 6375 were also oxidized by periodate. Rhamnose is a major component of fraction I from these two strains but in each case the rhamnose was stable to periodate, the oxidation of other sugars actually resulting in an increase in the rhamnose content of the remaining residue. This result suggests that either the rhamnose units are joined by 1 → 3 linkages or, alternatively, that two of the three hydroxyl groupings (on positions 2, 3, 4) of rhamnose are substituted.

DISCUSSION

The work described in the present paper was undertaken in an attempt to establish a relationship between components of the cell wall of *Lactobacillus casei* and the serological properties of the particular organism. The characteristic components of the cell wall of Gram-positive bacteria include mucopolysaccharides, mucopeptides and teichoic acids. Only some species of lactobacilli contain teichoic acid in the cell wall, although in others glycerol teichoic acid has been detected within the cells (Baddiley & Davison, 1961). Further, the presence of glycerol teichoic acid or ribitol teichoic acid in the wall could be related to the serological grouping of the particular organism, those in groups A and E containing glycerol and those in group D containing ribitol. Organisms of groups B, C and F contained no wall teichoic acid but an intracellular glycerol teichoic acid. This compound has been isolated from a strain of *L. casei* and its structure elucidated (Keleman & Baddiley, 1961). The results of Baddiley & Davison (1961) suggest that the teichoic acids participate in the serological reactions of some strains of lactobacilli, but not those of groups B and C, i.e. those designated *L. casei* or *L. casei* var. *rhamnosus*; in these organisms therefore another component of the cell wall must be associated with serological specificity.

Cummins & Harris (1956) studied the cell wall composition of lactobacilli (and other organisms) and their results showed that only two of the strains examined contained rhamnose, one designated *Lactobacillus casei* (NCIB 8079) and the other

L. delbrueckii (NCIB 7473). The latter has subsequently been shown to be a strain of *L. casei* var. *rhamnosus* (group C) so that it seemed likely that a knowledge of the occurrence of cell wall polysaccharides containing rhamnose might be valuable in the designation of organisms as *L. casei*. Examination of a laboratory strain 55 of *L. casei* showed that rhamnose was a major component of the wall and was, together with galactose and glucose, associated with the serological specificity (Knox & Brandsen, 1962). In the present investigation, cell wall components of five other strains of *L. casei* were studied, four of these having been used by Sharpe (1955*a*) to distinguish between organisms of groups B and C. To investigate the group specific products of these strains of *L. casei* the technique developed by McCarty was used. McCarty (1952*b*) incubated organisms of group A streptococci with *Streptomyces albus* enzyme and obtained soluble products containing the group specific carbohydrate. The preparation also contained the components of the cell wall mucopeptide, but subsequently it was shown that formamide would separate the polysaccharide from the mucopeptide (Krause & McCarty, 1961).

The analyses of the soluble products obtained from cell wall following digestion with the streptomyces muralytic enzyme are given in Table 1. Fraction I in each case was the product precipitated by 70% saturation with $(NH_4)_2SO_4$ (this was the concentration of $(NH_4)_2SO_4$ required to precipitate the enzyme), and fraction II was the material then remaining in the supernatant fluid. When the analyses for the wall and these two fractions from each of the four strains designated *Lactobacillus casei* are examined, three observations emerge: (i) for any one strain, the relative proportion of rhamnose and glucose in the two fractions is similar to that in the wall; (ii) each preparation of fraction II contains more hexosamine (glucosamine+galactosamine) and less muramic acid than the corresponding wall; (iii) from the analyses for nitrogen, fraction II preparations contain only a minor peptide component, whereas fraction I preparations contain less carbohydrate and a greater proportion of peptide. As it has also been observed that the diffusible fraction following enzyme action contains relatively large amounts of muramic acid, the results suggest the streptomyces enzyme is hydrolysing specific linkages in the cell wall mucopeptide. Krause & McCarty (1961) have reached the same conclusion from their studies on the action of the streptomyces enzyme on streptococcal cell wall.

With the two strains of *Lactobacillus casei* var. *rhamnosus* a different pattern is obvious. In each case fraction I differed significantly in composition from fraction II, fraction I having a much greater content of rhamnose and virtually no hexosamine or peptide component (following further fractionation from ethylene glycol with acetone). The difference is even more pronounced for strain NCIB 7473; fraction I contained mannose but not galactose, whereas galactose was a major component of fraction II and mannose only a minor component. The results indicate that the wall of strains of *L. casei* var. *rhamnosus* contains two polysaccharide components. One component, in fraction II, is almost devoid of rhamnose and is also present in strain NIRD R094 (group C). The other component, fraction I, contains most of the rhamnose, and it may be concluded that a polysaccharide composed of galactose, glucose and rhamnose represents about half the weight of the wall. The presence of this major cell wall polysaccharide in strains of *L. casei* var. *rhamnosus* is associated with a decrease in the mucopeptide fraction, the hexosamine, muramic acid

and nitrogen values being considerably less than those for the other group C organism, strain NIRD R094. Fractions I and II differ considerably in composition but from studies on the rate of dissolution of the wall (Fig. 1) there is no evidence for the two polysaccharide fractions being released at different rates; further, the rate of release was not affected by changes in pH between 5·3 and 8·0. There is no evidence, therefore, that enzymes of different specificity are acting on the cell wall to release the polysaccharide components. The small amount of hexosamine and nitrogen in preparations of fraction I may be indicative that this fraction is also released from the cell wall by hydrolysis of specific linkages in the mucopeptide.

From the analytical figures it is possible to calculate the percentage of the dry weight of the fractions which has been accounted for. This has been done for the six preparations of fraction II and the preparations of fraction I from the two strains of *Lactobacillus casei* var. *rhamnosus*. Assuming that the hexosamines are acetylated and that the total nitrogen is accounted for by amino acids and hexosamines, the percentage recovery lies between 91 and 98 %, with an average of 95 % (allowing for water of hydrolysis). Thus it seems unlikely that any of the preparations contain significant amounts of undetected components.

Qualitative and quantitative precipitin studies have confirmed the conclusion of Sharpe (1955*a*) that strains of *Lactobacillus casei* can be divided into two groups, subsequently designated B and C (Sharpe & Wheater, 1957). Rhamnose is the major component of the products obtained from group B organisms and it is also the most effective inhibitor of the precipitin reaction between the corresponding fraction II preparations and homologous antiserum. Further, the periodate oxidation of rhamnose residues in fraction II is accompanied by a decreased serological reactivity. Galactose and glucose contribute to the serological specificity of strain 55, and the cell walls apparently contain a polysaccharide component (isolated as fraction II*c*) in which these two sugars are important components. A fraction with similar properties has also been isolated from autolysed cell walls of strain 55 (Knox & Brandsen, 1962). However, when the analytical and serological results for the two strains NIRD DECP and NIRD H831 are compared, it is apparent that galactose and glucose could not be contributing to their cross reaction. It may therefore be concluded that the classification of organisms as 'group B' depends on specific carbohydrate components which contain rhamnose, with some strains (such as 55) containing galactose and/or glucose as additional antigenic determinants.

Group C organisms comprised one strain of *Lactobacillus casei* NIRD R094, containing only a small amount of rhamnose in the cell wall, and two strains of *L. casei* var. *rhamnosus*, in which rhamnose is a major component of the wall. Fractionation of the soluble products, released from each of the latter two strains by the streptomyces muralytic enzyme, showed most of the rhamnose was present in fraction I (precipitated by $(NH_4)_2SO_4$) whereas fraction II contained a much smaller amount of rhamnose and was very similar in composition to fraction II from *L. casei* NIRD R094. The preparations of fraction II from strains of *L. casei* var. *rhamnosus* cross react with antisera to NIRD R094 but do not give a precipitate with homologous antiserum, this reaction being given only by fraction I. It is concluded therefore that strains of *L. casei* var. *rhamnosus* cross react with strains of *L. casei* in group C because of the presence of one specific antigenic determinant with a low rhamnose to glucose ratio (in fraction II), whereas antibodies produced on

injection of these organisms are predominantly specific for another antigenic determinant with a greater rhamnose to glucose ratio (in fraction I). Sharpe (1955*a*) observed that cultures of *L. casei* var. *rhamnosus* grown at 37° stimulated the production of predominantly type serum, whereas with *L. casei* incubation at 37° gave organisms which favoured the formation of group antibodies. It therefore appears probable that fraction I contains the type specific product and fraction II the group specific product.

The major components of fraction II from group C organisms are galactose and glucose, but only glucose is a significant inhibitor of the precipitin test. Additional studies indicate that the specific determinant contains glucose joined to the adjacent sugar by a β linkage, the adjacent sugar probably being glucose substituted in the 6 position. Rhamnose is the major component of fraction I from the two strains of *Lactobacillus casei* var. *rhamnosus* but again glucose is the most efficient inhibitor. However, studies on the inhibition of glucosides gave results which do not clearly define the specificity in more detail, although it is probable that once again glucose is joined to the adjacent sugar by a β linkage. Periodate oxidation studies showed that the rhamnose units in fraction I from *Lactobacillus casei* var. *rhamnosus* and fraction II from *L. casei* group B are joined by different linkages, probably $1 \rightarrow 3$ for the former and either $1 \rightarrow 2$ or $1 \rightarrow 4$ for the latter. The results for the preparation from *L. casei* var. *rhamnosus* could also be accounted for by a highly branched structure in which every rhamnose unit is substituted on two of the three hydroxyl groups; such a structure, however, would appear less probable.

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The Serological Classification of Varieties of *Lactobacillus casei*

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SUMMARY

A comparison has been made between the serological properties of 35 strains of *Lactobacillus casei* and the carbohydrate components of corresponding cell-wall preparations. All strains of *L. casei* var. *rhamnosus* belonged to serological group C, whereas strains of *L. casei* var. *casei* belonged to group B or C. Rhamnose was the major cell-wall component of group B strains, galactose and glucose being minor and variable components. Of the group C strains only those classified as *L. casei* var. *rhamnosus* contained significant amounts of rhamnose in the cell wall. All strains in group C, however, contained galactose and glucose as major cell-wall components.

INTRODUCTION

The studies of Sharpe (1955*a*) and Sharpe & Wheater (1957) showed that strains of *Lactobacillus casei* belong to one of two serological groups, designated B and C and that group C also contained strains of *L. casei* var. *rhamnosus*. Subsequently soluble group-specific products were isolated from cell walls of the strains of *L. casei* and *L. casei* var. *rhamnosus* used by Sharpe, and their properties described (Knox & Brandsen, 1962; Knox, 1963). From a comparison of the chemical analyses and serological reactions of the preparations several conclusions were drawn. First, walls from strains of *L. casei* group B had a high rhamnose:glucose ratio and serological specificity was predominantly associated with rhamnose. Secondly, wall from the strain of *L. casei* group C had a low rhamnose:glucose ratio and serological specificity was predominantly associated with glucose. Thirdly, wall from two strains of *L. casei* var. *rhamnosus* had an intermediate rhamnose:glucose ratio; from both strains two products were isolated which were readily distinguishable serologically and chemically but in each glucose was responsible for serological specificity. To test whether these conclusions were generally applicable, a survey has now been made of the serological properties and cell-wall components of several strains of *L. casei* isolated from saliva (Featherstone, Murphy, Spies & Goldsworthy, personal communication).

METHODS

Organisms. Cultures of organisms used by Sharpe (1955*a*) were obtained from the National Collection of Type Cultures, Colindale, London (designated NCTC), the National Institute for Research in Dairying, Reading, England (NIRD) and the National Collection of Industrial Bacteria, Torry Research Station, Aberdeen, Scotland (NCIB). These organisms were *Lactobacillus casei* NIRD H831 and *L. casei*

NIRD DECP belonging to serological group B, and *L. casei* NIRD R094 and *L. helveticus* NCTC 6375 (*L. casei* var. *rhamnosus*) of group C. The other strains of *L. casei* which we have used had been classified by Mr J. L. Featherstone, Mr M. D. Murphy, Mr H. C. Spies and Dr N. E. Goldsworthy, who communicated their results to us and gave us cultures of their organisms: 24 were designated *L. casei* var. *casei* and 11 *L. casei* var. *rhamnosus*. The studies of various workers have shown that these two varieties account for 80–85% of the strains of *L. casei* (Davis, 1955; Rogosa *et al.* 1953; Featherstone, Murphy, Spies & Goldsworthy, personal communication).

Media. Cultures for inoculation into rabbits and for the preparation of HCl extracts were grown in the appropriate media described by Sharpe (1955*b*). The group sera used were those prepared for the previous investigation (Knox, 1963).

Methods. The procedures used for preparing and analysing cell-wall preparations have been described previously (Knox & Brandsen, 1962; Knox, 1963).

Precipitin tests. Qualitative precipitin tests were performed on acid extracts of organisms (Sharpe, 1955*a*) and on lysates of cell wall. Lysates were prepared by incubating samples of cell walls (10–12 mg. dry wt.) for 2 days at 37° with 0·5 ml. of streptomyces muralytic enzyme (McCarty, 1952*a*, *b*; Knox, 1963). Certain of these lysates were also examined by the Ouchterlony method (Ouchterlony, 1953).

RESULTS

Serological classification. Thirteen strains of *Lactobacillus casei* var. *casei* belonged to group B. Eleven strains of *L. casei* var. *casei* and all (11) strains of *L. casei* var. *rhamnosus* belonged to group C.

Cell-wall lysates were prepared from about half the strains in each category and in each case the serological classification was confirmed by qualitative precipitin tests and the Ouchterlony technique. The Ouchterlony test revealed that extracts of group B organisms contained only one component which gave a precipitate with antiserum to Lactobacillus NIRD R094. Serological identity was established between the product present in the extracts and purified fraction II from strain NIRD R094 (Knox, 1963). Extract of group C organisms contained a single component reacting with antiserum to strain NIRD H831; the product in the extracts was serologically identical with the purified fraction II isolated from strain NIRD H831.

Carbohydrate components of wall preparation

Carbohydrate chromatography. All preparations contained glucose. Also, all preparations contained galactose, except two from strains of *Lactobacillus casei* var. *casei*, group B. The 6-deoxyhexose component moved with the same R_F value as rhamnose.

Qualitative analyses. Analyses of six typical cell-wall preparations are given in Table 1. The analyses for organisms in each of the three categories are grouped in Table 2. The sum of the values for rhamnose, glucose and galactose lay between 32 and 42% for most strains of *Lactobacillus casei* var. *casei*. Where different figures were obtained the analytical results have been given separately. Nine strains of *L. casei* var. *rhamnosus* contained rhamnose and for eight of these the sum of the analytical values was 47–67%. Two strains of *L. casei* var. *rhamnosus* did not contain rhamnose; these results are given separately.

The molar ratio of rhamnose to glucose was calculated for each strain, and the results are shown in Table 3. The molar ratios of glucose to galactose varied over a wide range for strains in serological group B. With strains in group C, however, the molar ratio lay between 0·72:1 and 1:1 for 10 of the 11 strains of *Lactobacillus casei* var. *casei*, and the 2 strains of *L. casei* var. *rhamnosus* which did not contain rhamnose. In contrast only 3 of the 9 remaining strains of *L. casei* var. *rhamnosus* gave a value within this range, the ratios extending from 0·33:1 to 2·1:1.

Table 1. *Carbohydrate components of typical cell-wall preparations from varieties of* Lactobacillus casei

Variety	Strain (identification no.)	Serological group	Rhamnose (%)	Glucose (%)	Galactose (%)
casei	5	B	20	10	9
	21	B	19	15	8
	1	C	0	15	20
	16	C	0·5	18	22
rhamnosus	15	C	19	13	24
	110	C	27	23	12

Table 2. *Summary of cell-wall analyses from varieties of* Lactobacillus casei

Variety	Serological group	Total no. of strains	Rhamnose (%)	Glucose (%)	Galactose (%)	Total (%)
casei	B	11	18–28	4–13	0–9	32–42
		2	12,41	4, 4	3, 8	19, 53
casei	C	8	0–2·8 (6·7)*	14–18	15–23	31–45
		3	0–1·0	6–10	11–16	21–23
rhamnosus	C	8	16–29	12–23 (7)*	12–33	47–67
		1	26	7·5	7	41
		2	0·5, 0·5	16, 26	22, 29	39, 56

* For all except one strain the values lay within the range indicated. The remaining figure is given in brackets.

Table 3. *Molar ratios of rhamnose to glucose for the cell-wall preparations from varieties of* Lactobacillus casei

	L. casei		
	serological group B	serological group C	
Molar ratio	var. *casei*	var. *casei*	var. *rhamnosus*
< 0·10	0	6	2
0·10–0·31	0	5	0
1·1–1·7	4	0	7
2·2–3·0	4	0	0
3·1–4·5	3	0	2
5·5–7·5	1	0	0
9·1–19·8	1	0	0

DISCUSSION

In an extensive survey of more than 400 Lactobacillus strains Sharpe (1955*a*) classified 70 % of these strains into six serological groups. There was close agreement between these groupings and the physiological groups defined by Briggs (1953). In a subsequent discussion of the classification of Lactobacillus Rogosa & Sharpe (1959) indicated that most strains of *Lactobacillus casei* var. *casei* and *L. casei* var. *alactosus* belong to serological group B, the remainder belonging to group C; all strains of *L. casei* var. *rhamnosus*, however, belonged to group C.

The strains examined in the present work had been classified on the basis of physiological tests. All strains of *Lactobacillus casei* var. *rhamnosus* have been found to belong to serological group C whereas strains of *L. casei* var. *casei* belong to either group B or group C. The conclusion of Sharpe that there is a close agreement between serological and physiological groupings of Lactobacillus has therefore been confirmed.

The serological groupings depend on cell-wall components and previous studies have shown that the group-specific products isolated from the cell wall of strains of *Lactobacillus casei* var. *casei* and *L. casei* var. *rhamnosus* are predominantly polysaccharide. The quantitative analyses performed on cell-wall preparations from 35 additional strains of groups B and C indicate that polysaccharides are important components of the cell-wall preparations, the analyses totalling 31–45 % for 19 of the 24 strains of *L. casei* var. *casei*. In agreement with earlier results (Knox, 1963) the carbohydrate content of wall preparations from strains of *L. casei* var. *rhamnosus* were even higher—47–67 % for 8 of the 9 rhamnose-containing strains. Two strains of *L. casei* var. *rhamnosus* did not contain rhamnose as a cell-wall component; the analyses and serological properties are therefore those expected of a strain of *L. casei* var. *casei* of serological group C.

Table 3 illustrates the close relationship between serological groupings of the different organisms and the molar ratio of rhamnose to glucose. As observed previously, organisms belonging to serological group B are characterized by a high rhamnose:glucose ratio which is greater than 2:1 for a majority of the strains (9 of 13). Organisms in serological group C on the other hand are characterized by a lower rhamnose:glucose ratio—less than 2:1 for all but 2 of the 22 strains. Furthermore, rhamnose was only present as a major component in the wall preparations of strains of *Lactobacillus casei* var. *rhamnosus*; for all strains of *L. casei* var. *casei* the rhamnose content was very low.

Galactose and glucose are present as variable but minor components of preparations from group B organisms, an observation in agreement with the conclusion (Knox, 1963) that rhamnose is responsible for the serological specificity. Strains of group C organisms, however, contain greater amounts of galactose and glucose and, furthermore, for those strains which do not contain rhamnose, the molar ratios of glucose to galactose lie within the narrow range of 0·72:1 to 1:1. The variable glucose:galactose ratios for strains of *Lactobacillus casei* var. *rhamnosus* may be accounted for by variations in the relative amounts of the two specific polysaccharides previously shown to be present (Knox, 1963).

These results conform with those expected from the earlier studies on group-specific components, and certain generalizations now appear to be valid. First, all

strains of *Lactobacillus casei* var. *casei* of serological group B contain rhamnose as a major cell-wall component. Secondly, strains of *L. casei* var. *casei* of serological group C do not contain rhamnose, the major cell-wall carbohydrate components being galactose and glucose in about equimolar proportions. Thirdly, rhamnose is a major component of the cell wall of most strains of *L. casei* var. *rhamnosus*: these strains also belong to serological group C, for, as shown previously, rhamnose does not contribute to their serological specificity.

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The Fractionation of *Clostridium welchii* ε-Antigen on Cellulose Ion Exchangers

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SUMMARY

A low-protein medium is described for the production of reasonable yields of *Clostridium welchii* (*perfringens*) ε-prototoxin. The ε-antigen in young cultures on this medium was virtually non-toxic, 99·8% being in the form of prototoxin. Its purity, estimated in terms of the protein content of crystalline ε-prototoxin, was 50%. Fractionation of concentrates of the antigen on columns of diethylaminoethyl- and carboxymethyl-celluloses suggested that a number of proteins intermediate between ε-prototoxin and ε-toxin were present which differed from the latter in their isoelectric points and in their activation ratios. The concentration and number of these intermediates increased as the incubation of cultures was prolonged. Since they could be fully converted to ε-toxin by trypsin it is probable that they were formed from ε-prototoxin by proteolytic or spontaneous degradation. In toxicity tests, death caused by unactivated antigen may be delayed. This evidence suggested that the intermediates were intrinsically non-toxic but could become activated in the animal body. The activation ratios might then be regarded as a measure of the ease with which the intermediates could be converted to toxin *in vivo*. Physical data and the amino acid composition of crystalline ε-prototoxin are presented.

INTRODUCTION

Of the several toxins and enzymes found in cultures of *Clostridium welchii* (*perfringens*), only two, epsilon (ε) and iota (ι), are known to be produced as non-toxic precursors. Epsilon toxin is produced predominantly by *C. welchii* type D and to a lesser extent by type B. There is no doubt that the enterotoxaemia and death associated with disease due to type D strains are the result of epsilon intoxication (Bennetts, 1932; Bullen & Scarisbrick, 1957). Bosworth & Glover (1934–35) were the first to show that culture filtrates of *C. welchii* type D could be 'activated', that is, their toxicity markedly enhanced, by incubation with intestinal contents. This was found to be due to tryptic activity. Turner & Rodwell (1943) extended these observations and used the term 'prototoxin' to denote the precursor. They also showed that proteases other than trypsin were capable of activating type D filtrates. More recently, Meisel, Albricht & Rymkiewicz (1960) extracted a non-toxic prototoxin by ultrasonic lysis of type D vegetative organisms.

For the sake of clarity, three terms to be used in this paper may be defined: (1) ε-prototoxin is by definition non-toxic, and is a specific entity which becomes ε-toxin when it is treated with trypsin or certain other proteases. (2) ε-toxin is

completely toxic; its toxicity cannot be further increased by proteases. (3) ϵ-antigen is used as a general term to describe prototoxin, toxin or toxoid or a mixture of any of these. ϵ-Prototoxin, toxin and toxoid stimulate the production of antitoxin which reacts observably with them all. Differences exist in the abilities of the different antigens to stimulate antibody production and in their power to combine with antibody. These differences are usually imperceptible when the antigen is estimated by conventional flocculation with antitoxin.

Orlans, Richards & Jones (1960) worked on the purification and properties of ϵ-toxin; their purified material had an activation ratio of two, i.e. was a mixture containing equal amounts of toxin and prototoxin. The highest toxicity they obtained by activation with trypsin was 28 m.l.d./Lf. This indicates that a large proportion (80–90 %) of the purified toxin may have consisted of spontaneously produced toxoid, since a fully activated toxin usually contains 200–300 m.l.d./Lf. Verwoerd (1960) crystallized prototoxin after methanol fractionation. This is known to be a rather destructive procedure unless extreme care is taken, but the toxicities he obtained after trypsin treatment were much higher than those published by the previous authors. Activation ratios were not determined, and it is therefore impossible to establish the ratio of prototoxin to toxin in his crystals. The work of Meisel *et al.* (1960) has been repeated in these laboratories with some success. Non-toxic lysates from *Clostridium welchii* type D organisms were obtained which became toxic on activation. However, the amount of prototoxin in these lysates was very small and spontaneous activation occurred as they were being worked up (Mrs I. Batty, personal communication). The present paper describes the production of ϵ-antigen in a low-protein medium and the results obtained when concentrates were fractionated on columns of cellulose ion exchangers. The method of purification and crystallization of ϵ-prototoxin finally adopted was described in a preliminary note (Thomson, 1962). Certain properties of this crystalline material are reported here.

METHODS

Production of antigen. To facilitate purification of antigen, a low-protein medium was devised. This had the following composition: pancreatic digest of Ca caseinate, 400 ml.; yeast dialysis diffusate, 50 ml.; cyst(e)ine, 0·2 g.; glucose, 2·5 g.; thiamine, 0·5 mg.; riboflavin, 0·5 mg.; pyridoxin, 0·5 mg.; Ca pantothenate, 2 mg.; $CaCl_2$, 2 g.; $NaHCO_3$, 2 g.; water to 1 l. This solution was adjusted to pH 7·5 before autoclaving for 15 min. at 115°. The Ca caseinate digest was prepared by the method of Gladstone & Fildes (1940). It contained about 10–12 g. total-N and about 4 g. amino-N/l. as determined by the copper method of Pope & Stevens (1939). The yeast dialysis diffusate was prepared by dialysing a slurry of dried baker's yeast (Distillers Co. Ltd.) against water for 7 hr. at 80° in the proportions, 1·5 l. water/100 g. yeast, this diffusate being used in quantities as above.

The strain of *Clostridium welchii* type D used produced traces of α and κ toxins. No measurable quantities of λ or μ toxins were found.

Cultures were grown for 6–24 hr. in 15 l. bottles, and were harvested, after adding thiomersalate to 0·01 %, by sucking the fluid through a pad of Hyflo Supercel (Johns-Manville and Co. Ltd.) prepared on a lint base in a Buchner funnel.

Since ε-prototoxin is slowly activated by bacterial proteases and perhaps other factors, concentration and fractionation procedures were done at 5°. Fractions were stored at 2°.

Concentration of antigen. Culture filtrates prepared as above were concentrated by precipitation with ammonium sulphate (300 g./l.) and concentrated further by dialysis against polyethyleneglycol M.W. 15,000 ('Carbowax', Union Carbide Ltd., London, W. 1; Kohn, 1959). Seitz filtration through sterilizing (SB) mats (T. B. Ford Ltd., Loudwater, Bucks., England) removed fine particulate matter without loss of antigen.

Column fractionation. ε-Antigen concentrates were dialysed against appropriate buffers, applied to ion exchange columns, and fractions obtained by gradient elution. Columns were prepared with either diethylaminoethyl (DEAE)-cellulose or carboxymethyl (CM)-cellulose (DE 50 and CM 70, Whatman), anion and cation exchangers, respectively. For large-scale work involving about 2×10^6 Lf of antigen, 15 g. of dry ion exchanger were equilibrated with the required buffer. Fines were removed by several filtrations through lint and the remainder used to prepare a column 15 cm. × 2·5 cm. diameter. After loading with antigen, the column was eluted under gravity flow with linear pH, salt, or pH + salt gradients at a rate of 5 ml./hr./cm.^{2} surface area. Five, ten or twenty-five ml. fractions were collected. Antigen and protein were estimated in these fractions and in pooled groups of fractions. Protein concentrations were estimated by the modified Folin-Ciocalteu method of Lowry, Rosebrough, Farr & Randall (1951) with a Hilger 'Biochem Absorptiometer' and a filter transmitting at 580 mμ.

Protein-nitrogen (*protein-N*). Determinations were made in triplicate by the micro-Kjeldahl method, with 5% (w/v) trichloroacetic acid as protein precipitant. Digestion with 98% H_2SO_4 was assisted by a copper/selenium catalyst. The Markham apparatus (Markham, 1942) was used for distillation.

Activation of antigens. To obtain some measure of the relative proportions of ε-prototoxin and toxin in different fractions, samples were activated with trypsin. For most samples taken at different stages of purification the following procedure was satisfactory. The sample was diluted in nutrient broth at pH 7·3 to 10 Lf/ml. To this was added 2% (v/v) crude ox pancreatic extract (Gladstone & Fildes, 1940) or 0·005% (w/v) crystalline trypsin and the mixture incubated for 30 min. at 37°. Activated fractions were immediately cooled and stored at 2°. Toxicity tests were usually done on the same day.

Combining power determinations. (1) *In vitro* flocculation. The flocculating capacity of activated or unactivated samples against standard antiserum was determined as follows. A series of tubes containing constant volumes of a suitable dilution of sample and a graded series of volumes of antiserum were incubated in a water bath at 50°. The neutral mixture was the first to flocculate. The combining power (flocculation) was expressed as Lf/ml. (2) *In vivo* tests. Amounts of activated antigen increasing in steps of 10% were mixed with 10 units of antitoxin and held at room temperature for 30 min. The mixtures were then injected intravenously into pairs of mice (each 18–22 g.). The mixture containing the least volume of antigen which caused death in one out of two mice within 2 days was taken as the end-point. This was the L+ dose at 10 units. Results were recorded as 10 times the reciprocal of the L+ dose at 10 units and expressed as unit equivalents (u.e.)/ml.

Toxicity tests. Determinations were made before and after activation. Samples (0·5 ml.) of a series of dilutions differing by increments of 50 % were injected intravenously into pairs of mice. The highest dilution which caused death in both mice within 4 days was taken as the minimum lethal dose (LD 100) of the sample. For ease of comparison the reciprocal of the LD 100, i.e. LD 100/ml. has been used in tabulating results.

Specific toxicity. This is defined as the ratio of LD 100/ml. (activated) to Lf/ml. of the activated sample, and is expressed as LD 100/Lf.

Activation ratio. The ratio LD 100/Lf (activated):LD 100/Lf (unactivated) is the activation ratio of the sample.

RESULTS

The results obtained at different times during growth in the casein digest medium are shown in Table 1. The Lf/mg. protein-N, which indicates the purity of the antigen in the fraction, decreased as incubation proceeded. By comparison with the purity of crystalline prototoxin, the antigen in the 8 hr. culture accounted for 50 % of the protein. Even after 120 hr., 30 % of the protein was accounted for as

Table 1. *Changes in purity, combining power and activation ratios of ε-antigen with age of culture of* Clostridium welchii *type D*

Age of culture (hr.)	Combining power		Protein-N (mg./ml.)	Lf/mg. protein-N	LD 100/ml.		Activation ratio	Specific toxicity (LD 100/Lf)
	Lf/ml.	L+ (u.e./ml.)*			Unactivated	Activated		
6	105	78	0·016	6500	42	2×10^4	500	200
24	215	—	0·042	5070	200	$4{\cdot}3 \times 10^4$	200	200
54	250	—	0·058	4300	250	5×10^4	200	200
120	225	195	0·056	4000	225	$2{\cdot}3 \times 10^4$	100	100

* u.e. = Unit equivalents.

specific ε-antigen. The activation ratios were much higher than those found when routine high protein media were used. After 120 hr. the activation ratio was still 100, indicating that the antigen consisted 99 % of prototoxin and 1 % of toxin. In a meat broth medium the antigen is usually fully activated after 120 hr., and even after 24 hr. the ratio is seldom more than 50. The final column in Table 1 shows specific toxicities. The value of 200 for the first three samples was normally expected during this work. After 120 hr., however, it had decreased to half this value, indicating spontaneous toxoiding during the prolonged incubation period.

Concentration and purification of ε-antigen. Culture filtrates were precipitated with ammonium sulphate at 300 g./l. Recoveries of 90–100 % were regularly obtained and the purity of the antigen increased by a factor of about 1·5. As expected, virtually no loss of antigen or increase in purity occurred during further concentration by dialysis against polyethyleneglycol, nor did any fraction of the polymer dialyse into the antigen concentrate. During the concentration of filtrates it was found that, despite working at 5°, a considerable decrease in activation ratios occurred. For example, a 24 hr. culture filtrate that had an activation ratio of 100 when harvested had a ratio of 10 after concentration. During the concentration procedure the specific toxicity (LD 100/unit of combining power) remained un-

changed, indicating that there was no loss in toxicity or potential toxicity. The decrease in activation ratio must, therefore, have been due to a conversion of 9% of prototoxin to toxin. With filtrates taken after shorter growth periods the conversion was much less.

Fractionation on diethylaminoethyl (DEAE)-cellulose

Concentrates from cultures grown for different periods of time were fractionated.

Growth for 24 hr. Figure 1 shows the distribution of protein obtained by gradient elution from 0·005 M-Na phosphate (pH 7·0) to 0·4 M-Na phosphate (pH 5·9).

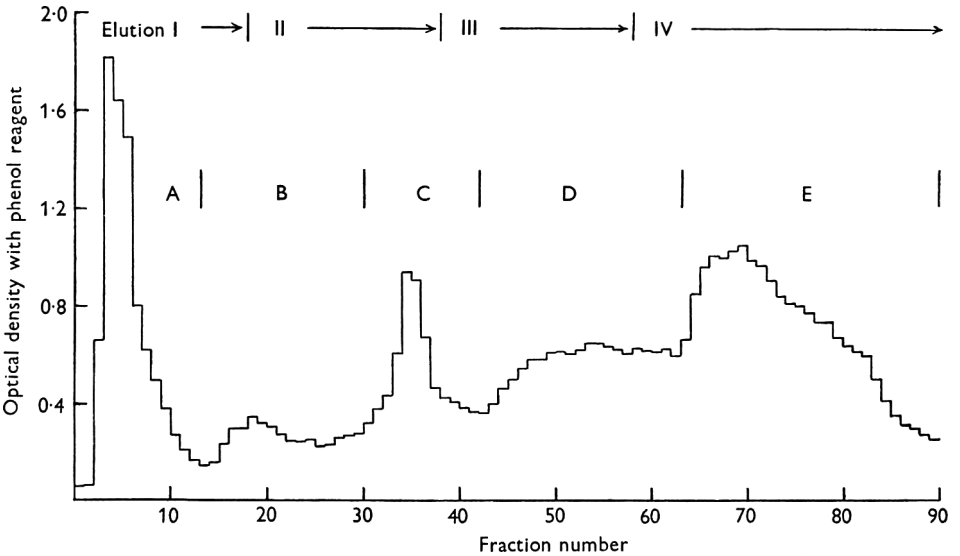

Fig. 1. Fractionation of a 24 hr. culture filtrate on DEAE-cellulose equilibrated at pH 7·0 with 0·005 M-Na phosphate. I. Elution with 0·005 M-phosphate pH 7·0. II. Gradient elution; mixing vessel, 550 ml. 0·005 M-phosphate pH 7·0, reservoir, 500 ml. 0·02 M-phosphate pH 6·1. III. Reservoir, 500 ml. 0·1 M-phosphate pH 5·9. IV. Reservoir, 0·4 M-phosphate pH 5·9. 25 ml. fractions collected. Bulked fractions A–E.

Fractions were analysed for protein and for antigen titre (Lf) and groups of fractions were bulked as shown in Table 2, which gives the analytical data for the bulked fractions. Antigen titres followed closely the protein concentration and ε-antigen was present in all fractions. There was an overall recovery of antigen of 90%. Fraction A, containing the highest percentage of antigen, and having the highest activation ratio, came through with the starting buffer and was not retarded in the column. As elution proceeded, the activation ratios of fractions leaving the column decreased steadily and at the same time there was a decrease in specific toxicity, indicating that toxoid was present in the later fractions.

Growth for 8 hr. The same elution technique was used and a similar quantity of antigen was fractionated as for the 24 hr. growth. The pattern obtained for protein distribution, shown in Fig. 2, was strikingly similar to that shown in Fig. 1. Despite this, the antigen distribution was quite different. Fractions 3–6 contained 99% of the antigen applied to the column, compared with 42% in the similar fraction in

Table 2. *Recovery of antigen, purities, toxicities and activation ratios of bulked fractions obtained by separation of a 24 hr. culture filtrate of* Clostridium welchii *type D concentrate on DEAE-cellulose. See Fig.* 1

	Vol. (ml.)	Combining power: Lf/ml.	Combining power: Recovery (%)	L+ (u.e./ml.)*	Protein-N (mg./ml.)	Lf/mg. protein-N	LD 100/ml.: Unactivated	LD 100/ml.: Activated	Activation ratio	Specific toxicity (LD 100/Lf)
Concentrate	40	24,000	—	21,000	3·06	7,850	$1–2·5 \times 10^5$	$1–2·5 \times 10^6$	10	40–100
Bulked fraction A	260	1,560	42	1,380	0·131	11,900	3×10^3	$1·5–3 \times 10^5$	50–100	100–200
B	410	115	5	85	0·021	5,400	450	$1–2 \times 10^4$	50–100	100–200
C	280	430	12·5	300	0·036	11,900	4×10^3	4×10^4	10	100
D	490	330	17	285	0·035	9,500	3×10^3	3×10^4	10	100
E	720	170	13	114	0·053	3,200	$0·7–1·7 \times 10^3$	$0·7 \times 10^4$	4–10	40

* u.e. = Unit equivalents.

Table 3. *Recovery of antigen, purities, toxicities and activation ratios of bulked fractions obtained by separation on CM-cellulose of an* 8 *hr. culture filtrate concentrate of* Clostridium welchii *type D after adsorption with DEAE-cellulose. See Fig.* 3

	Vol. (ml.)	Lf/ml.	Recovery (%)	Protein-N (mg./ml.)	Lf/mg. protein-N	LD 100/ml.: Unactivated	LD 100/ml.: Activated	Activation ratio	Specific toxicity (LD 100/Lf)
Concentrate	10	66,000	—	5·7	11,600	$1·3 \times 10^5$	$1·3 \times 10^7$	100	100
Bulked fractions D	115	250	4·5	0·027	9,250	$1–2·5 \times 10^3$	$1–2·5 \times 10^4$	10	40–100
F	50	1,340	10	0·119	11,250	$2·5–5 \times 10^3$	$1·25–2·5 \times 10^5$	50	100–200
H	45	900	6	0·074	12,100	$2·0–4·0 \times 10^3$	2×10^5	50–100	200
K	80	2,130	26	0·182	11,700	2×10^3	$4–8 \times 10^5$	200–400	200–400

Fig. 1. These fractions when bulked had an activation ratio of 100 and a purity of 11,700 Lf/mg. protein-N. They came through the column in the starting buffer.

Growth for 6 hr. Fractionation of these concentrates was similar in every way to that shown in Fig. 2; antigen was washed through by the starting buffer, its activation ratio was 500 and purity 13,000 Lf/mg. protein-N.

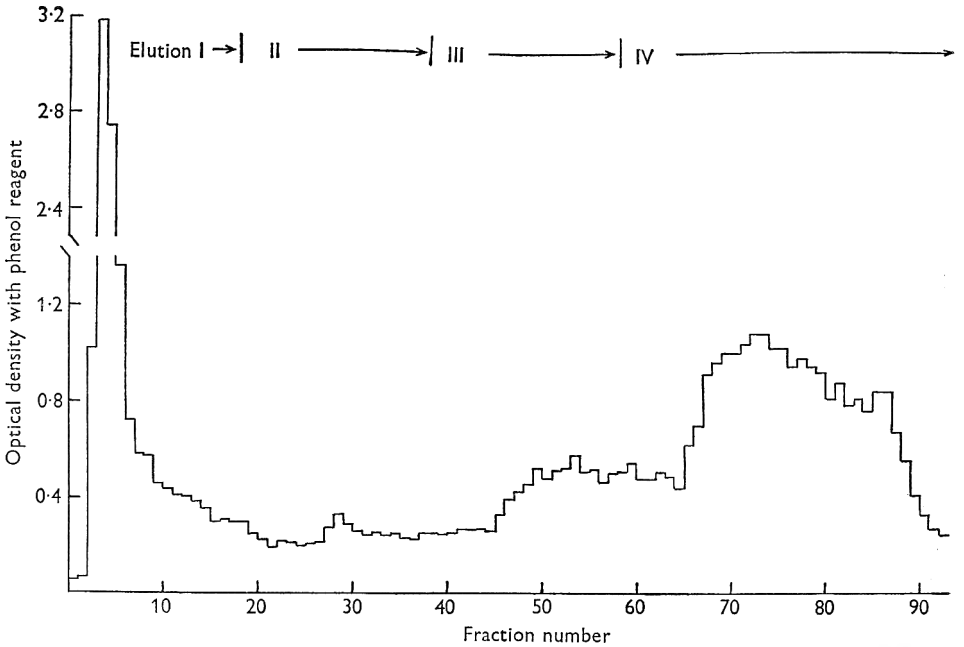

Fig. 2. Fractionation of an 8 hr. culture filtrate on DEAE-cellulose equilibrated at pH 7·0 with 0·005 M-Na phosphate. Gradient elution as in Fig. 1. 25 ml. fractions collected. Pooled fractions 3–6 = fraction A.

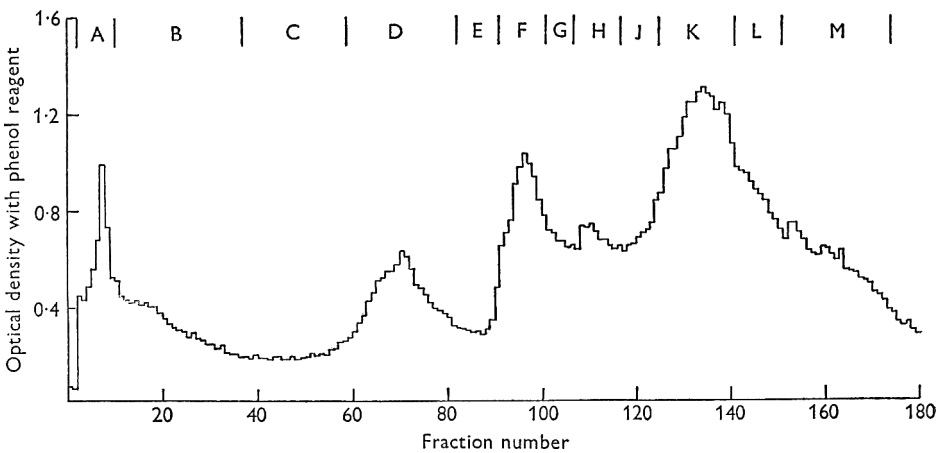

Fig. 3. Fractionation of Fraction A (Fig. 2) on CM-cellulose equilibrated at pH 6·0 with 0·005 M-Na phosphate. Gradient elution; mixing vessel, 550 ml. 0·005 M-phosphate pH 6·0; reservoir, 0·005 M-phosphate pH 7·2. 5 ml. fractions collected.

Fractionation on carboxymethyl-cellulose

Growth for 8 hr. Fraction A (pooled fractions 3–6) from the separation illustrated in Fig. 2 was concentrated with 'Carbowax' and fractionated on CM-cellulose. As seen in Fig. 3, the first fraction came through with the starting buffer. With this fraction no antigen was indicated by flocculation at any level of testing, but toxicity tests on activated samples showed traces of toxin. When estimations for protein were made on this fraction, heavy precipitation occurred with the phenol reagent, and optical densities were measured on the clear supernatant fluids after centrifuging. The cause of this interference was not pursued, but because of it one must suspect the accuracy of these measurements.

All fractions apart from the first contained considerable quantities of antigen; analytical data for the main fractions after bulking are given in Table 3. The distribution of activation ratios is of interest. As one would expect, the pattern obtained by fractionation on DEAE-cellulose (Table 2) was reversed, i.e. there was an increase in activation ratio as the pH value of elution increased; a similar increase in specific toxicity occurred. Antigen could be measured in fractions D–M, the total amount recovered accounting for 81 % of that applied to the column.

Growth for 6 hr. The fraction containing the antigen obtained from DEAE-cellulose column was concentrated and applied to a CM-cellulose column. The result of pH gradient elution is shown in Fig. 4. Under the conditions used, only two fractions were obtained, the first containing no antigen and behaving like fraction A in Fig. 3. The second fraction, containing the antigen, had an activation ratio of 500, and a purity of 12,500 Lf/mg. protein-N. When this fraction was concentrated to 10^5 Lf/ml. by dialysis against 'Carbowax' the antigen precipitated as blunt needle-like crystals.

Properties of crystalline ε-prototoxin

Ultracentrifugal analysis. A freeze-dried preparation of crystalline ε-prototoxin was dissolved in and equilibrated against 0·067 M-NaK phosphate (pH 7·0) and analysed in a Spinco Model E Ultracentrifuge. Use was made of the synthetic boundary cell to determine diffusion coefficient and of the Archibald technique of approach to equilibrium to determine weight average molecular weight. All runs were at 20°, and in determining the concentration the value of 0·186 ml./g. was taken as the refractive increment of the protein.

The partial specific volume, $\bar{V}$, was determined by measuring the weights of volumes of solution, solvent, and water delivered accurately with an Agla micrometer syringe (Burroughs Wellcome and Co. Ltd., London). By this method $\bar{V} = 0{\cdot}729$ ml./g.

The sedimentation coefficient corrected to water $S_{20°}W$ was 2·48S at a concentration of 0·00696 g./ml. The apparent diffusion coefficients, determined from the boundary spread in a sedimentation velocity run (Schachmann, 1957), indicated that there was some decrease with time, possibly due to dependence of $S_{20°}$ on concentration. Polydispersity would be indicated by an increase of apparent diffusion coefficient with time.

The diffusion coefficient obtained from the synthetic boundary-cell run corrected to water was $6{\cdot}76 \times 10^{-7}$ cm.²/sec. The weight average molecular weight, determined

from approach to equilibrium data (Trautmann & Crampton, 1959), was 40,500. From these data it was possible to show that the sample of protein used contained 92·5% material of that molecular weight. The molecular weight of contaminating protein was not greater than 14,000.

From the molecular weight, partial specific volume, and sedimentation coefficient, the ratio of the frictional coefficient of the molecule to that of an unsolvated sphere, f/f_s, was calculated to be 1·68. This represents a considerable degree of asymmetry

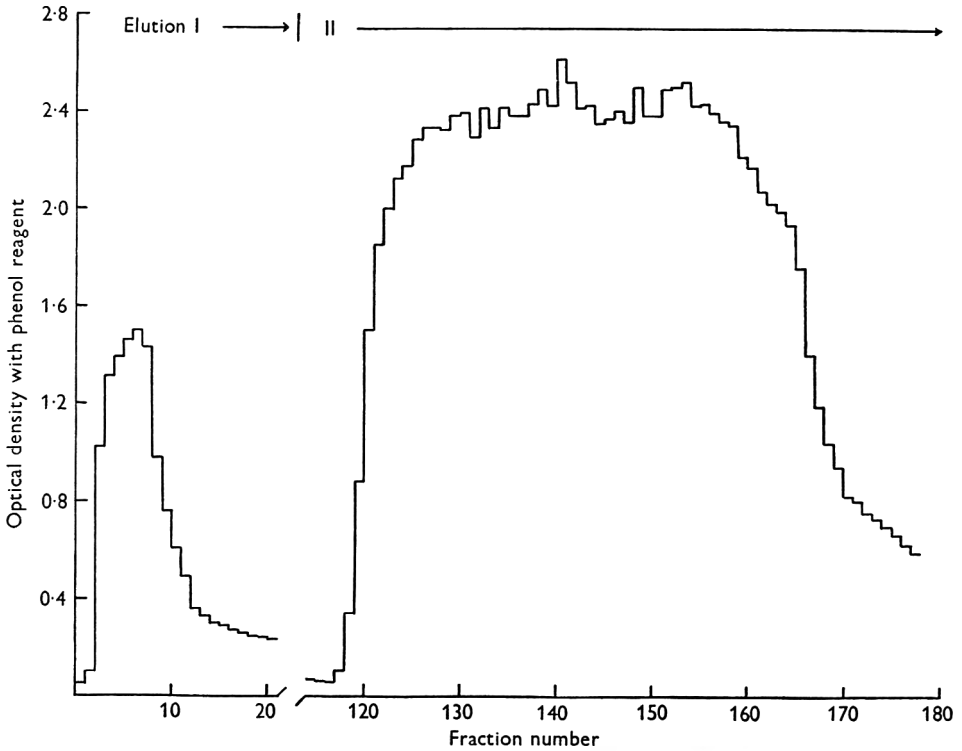

Fig. 4. Fractionation on CM-cellulose of antigen from a 6 hr. culture filtrate not adsorbed by DEAE-cellulose. CM-cellulose equilibrated at pH 6·0 with 0·005M-Na phosphate. I. Gradient elution; mixing vessel, 550 ml. 0·005M-Na phosphate pH 6·0; reservoir, 0·005M-phosphate pH 7·0 till fraction No. 82. II. Reservoir, 0·005M-phosphate pH 7·5, 10 ml. fractions collected.

Table 4. *Amino acid composition of crystalline ε-prototoxin of* Clostridium welchii *type D*

	%		%
Lysine	14·20	Alanine	3·00
Histidine	1·28	Valine	6·95
Ammonia	2·13	Methionine	1·92
Arginine	2·55	Isoleucine	3·71
Aspartic acid	17·30	Leucine	5·80
Glutamic acid	9·95	Tyrosine	6·02
Threonine	10·00	Phenylalanine	1·72
Serine	6·07	Tryptophan	0·53
Proline	3·80	Cyst(e)ine	0·59
Glycine	3·07		

and/or hydration. Assuming a hydration of 30%, the ratio corresponds to that of a prolate ellipsoid having an axial ratio of 9 (Oncley, 1941).

Amino acid analysis. The amino acid composition of crystalline ϵ-prototoxin was determined after hydrolysis with hydrochloric acid by using a Spinco Model 120 amino acid analyser (Spackman, Stein & Moore, 1958). Cyst(e)ine was determined after oxidation to cysteic acid with performic acid by the method of Hirs (1956). Tryptophan was estimated by the ultraviolet absorption procedure of Holiday (1936). The results are given in Table 4.

DISCUSSION

By definition ϵ-prototoxin is non-toxic and becomes toxic when acted upon by certain proteolytic enzymes. There is no doubt that activation is most rapid in the presence of these enzymes but it is possible that some activation may occur slowly in their absence. In culture filtrates which contained no proteases demonstrable by azocoll digestion (Oakley, Warrack & van Heyningen, 1946) or gelatin liquefaction, there was a considerable decrease in activation ratios during incubation, and even highly purified fractions of ϵ-antigen increased in toxicity on standing. ϵ-Prototoxin may therefore be considered a labile protein in so far as its conversion to toxin is concerned, its activation, either spontaneously, or by minute traces of enzyme, being easily shown.

The adsorption of proteins by ion exchangers depends on the net charge of the protein molecule, which in its turn is a function of the pH value of the solution and the isoelectric point of the protein. In fractionations on the anion exchanger DEAE-cellulose (Fig. 1) those fractions which were eluted first had higher isoelectric points than those eluted later. This relationship between the order of elution and the isoelectric point is reversed when a cation exchanger such as CM-cellulose is used. It can be seen from Tables 2 and 3 that there was a similar decrease and increase in the activation ratios of fractions as they were eluted from DEAE- and CM-cellulose, respectively, indicating that the activation ratio varied with the isoelectric point. To extrapolate, prototoxin would have the highest and toxin the lowest isoelectric point.

The similarity between the fractionations illustrated in Figs. 1 and 2 has already been noted. The main difference was that with the 8 hr.-growth filtrate (Fig. 2) only one fraction contained ϵ-antigen, whereas the other fractions contained non-specific protein only. However, in the 24 hr. filtrate (Fig. 1) ϵ-antigen was distributed over the whole series of fractions. If the non-specific proteins were still present in cultures after 24 hr. they would be expected to appear in the equivalent fractions from the 24 hr. filtrate. This is borne out (Table 1) by the lower purities of antigen in these fractions. However, all fractions shown in Figs. 1 and 3 had some toxicity. It is difficult to explain this in terms of net charge since ϵ-toxin is assumed to be a discrete entity. Perhaps ϵ-toxin was adsorbed by these various fractions and more adsorption occurred with proteins having a lower isoelectric point. This would account for the decrease in activation ratio (or increase in toxicity/unit of combining power) of fractions as they were eluted from the column. This explanation is, however, rendered unlikely by the fact that in Fig. 2 no toxin accompanied a similar spread of fractions.

Estimations of the toxicity of ε-antigen fractions were performed by the intravenous injection of dilutions of the fraction into mice which were then observed for 4 days for signs of intoxication. Normally a lethal dose of ε-toxin from an activated filtrate will kill in less than 24 hr. and an assessment of the toxicity of activated samples can be made in 1 day. With unactivated samples, however, death is often delayed for a day or more.

The results obtained in the estimation of unactivated antigen may be explained by assuming that the injected antigen undergoes slow activation *in vivo*, the toxicity of the antigen being thus determined by the ease with which it can be activated, as well as by the amount of toxin *per se* in the antigen. The evidence obtained by ion exchange fractionation indicates that the original crude antigenic complex is composed of a number of fractions differing slightly in net charge. If these fractions are stepwise degradation products of prototoxin, that is, intermediates in the conversion of prototoxin to toxin, then the ease by which they are further degraded to toxin *in vivo* will depend upon the amount of degradation they have already undergone. The activation ratios of these fractions will therefore indicate the degree of degradation, those with the highest activation ratio being the least degraded. But it has been previously shown that the activation ratios of fractions bear a direct relationship to the isoelectric points. This means that as the prototoxin is degraded to toxin there is a gradual decrease in isoelectric point. This could be accounted for by a decrease in the number of basic groups on the molecule. The activation of prototoxin to toxin is therefore envisaged as a stepwise removal from the prototoxin molecule of basic amino acids or of peptides containing these. As the prototoxin is being activated it remains non-toxic, but the ease by which it can be converted to toxin *in vivo* is increased, the result being that there is a decrease in the activation ratios, and an increase in apparent toxicity due to *in vivo* activation of intermediates formed during activation.

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Morphological Response of *Trichophyton mentagrophytes* to Methionine

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SUMMARY

Granular cultures of *Trichophyton mentagrophytes* changed their growth habit when grown on a medium containing, as the source of nitrogen, the 19 L-amino acids found in hydrolysed casein, a response not seen when methionine was omitted. In contrast, pleomorphic cultures of *T. mentagrophytes* grown on amino acid medium were not modified. The methionine effect has been used successfully to differentiate between normal granular cultures and mutant pleomorphic cultures. Also, isolates characterized as atypical pleomorphs could be classified as metabolically granular or pleomorphic.

INTRODUCTION

Sabouraud (1910) described a striking morphological alteration in old cultures of dermatophyte fungi where tufts of downy or woolly white mycelium grew on the surface of the parent granular culture. Isolated tufts remained fluffy and did not revert to the parental type. Sabouraud termed these alterations pleomorphic and considered that they represented 'fixed mutations'. He observed that all studied species of Trichophyton formed pleomorphs which lost the ability to form macroconidia and chlamydospores, although all continued to produce microconidia. A working definition of a pleomorphic mutant based on Sabouraud's original and still valid observations must include a description of the surface growth as fluffy or downy, the loss of ability to form pigment, and sporulation only as microconidia. Granular cultures of *Trichophyton mentagrophytes* (Robin) Blanchard differ markedly from pleomorphic cultures in that the surface growth is appressed and 'grainy' in appearance, both surface and reverse sides of the colony are pigmented, and macro- and microconidia as well as chlamydospores are formed. Although Sabouraud reported that all pleomorphic cultures looked the same, Robbins & McVeigh (1949) described a series of pleomorphic isolates from a granular culture of *T. mentagrophytes* which varied in their modes of surface growth. Thus, a morphological description alone may not be sufficient to describe pleomorphism when there are gradations or degrees of alteration. As part of a project to elucidate the metabolic and genetic basis for the observed growth patterns in pleomorphism, the responses of granular and pleomorphic cultures of *T. mentagrophytes* to a variety of metabolic and physical stresses are being examined. The work to be reported describes one such metabolic stress, i.e. a culture medium containing methionine which successfully distinguishes between typical granular and typical pleomorphic cultures. Based on the type of morphological response to methionine, isolates otherwise intermediate in appearance can be classified as metabolically granular or pleomorphic.

METHODS

Organisms. Cultures of *Trichophyton mentagrophytes* were obtained from Dr W. J. Robbins (New York Botanical Garden) and Dr L. Ajello (Communicable Disease Centre, Atlanta, Georgia). Cultures of several dermatophyte fungi were obtained from Dr E. O'Hern (Downstate Medical Centre, Brooklyn, New York) and the Department of Microbiology and Immunology (Albert Einstein College of Medicine).

Culture media. All media used were identical except for the nitrogen source. The basal medium contained (g. or ml./l.): $MgSO_4.7H_2O$, 0·5; KH_2PO_4, 1·5; K_2HPO_4, 0·15; trace element solution, 0·5; thiamine stock (1 mg./ml.), 10; glucose, 20; agar, 15. Stock medium contained Difco Neopeptone at 0·2% (w/v). Experimental media contained one of the following nitrogen sources: (1) Vitamin-free acid hydrolysed casein (General Biochemicals Co. or Nutritional Biochemicals Corp.), 20 ml./l. to give a final concentration of 2 mg. casein/ml.; tryptophan (40 μg./ml.) was added to casein medium since acid hydrolysis destroys the tryptophan normally present; (2) 19 L-amino acids (California Corporation for Biochemical Research) each at the same concentration as found in acid hydrolysed casein (including tryptophan) to give a final concentration of 1·76 mg. amino acids/ml. Analysis of the hydrolysed casein (personal communication, Dr W. J. Robbins) used here differs somewhat from that of casein published (McMeekin, 1954); hence, it was felt worth while to give the analysis here. Twenty ml. hydrolysed casein contained MM.: alanine, 0·43; arginine, 0·44; aspartic acid, 0·62; cystine, 0·025; glutamic acid, 2·97; glycine, 0·13; histidine, 0·32; hydroxyproline, 0·03; iso-leucine, 0·75; leucine, 0·96; lysine, 0·74; methionine, 0·46; phenylalanine, 0·47; proline, 1·56; serine, 0·095; threonine, 0·47; tryptophan, 0·22; tyrosine, 0·72; valine, 1·35. The 19 L-amino acid medium was also used when various analogues and homologues of methionine (Nutritional Biochemicals Corp.) were tested. All media were adjusted to pH 5·7.

Conditions of growth. Stock cultures were grown in test tubes on Neopeptone medium and stored at 5°. Cultures used as a source of inoculum were grown in Petri dishes on Neopeptone medium at 26°. Inocula for all experiments were disks of agar cut from the advancing edge of mycelial growth with a sterile cork borer (4 mm. diameter) and placed, one disk/plate, at the centre of the experimental agar medium. All cultures were incubated at 26° for 2 weeks at which time observations were made.

RESULTS

Macroscopic appearance. When grown on amino acid medium, *Trichophyton mentagrophytes* (M12-4, NYBG) had an uneven irregular advancing hyphal edge (Pl. 1, fig. 1) contrasting with the smooth edge on hydrolysed casein medium; but a typical pleomorphic culture (T16-12, NYBG) did not change on the 19 L-amino acid medium (Pl. 1, figs. 3, 4). When methionine was omitted from the amino acid medium there was no morphological response by the granular culture (Pl. 1, fig. 2); when each of the other amino acids was omitted singly, with methionine present, there was still a positive response. When subcultured from amino acid medium to Neopeptone or casein medium, the growth pattern of the granular culture immediately returned to normal. On amino acid medium, the rates of growth of the granular and pleomorphic cultures were slightly lower with than without methionine.

Microscopic appearance. Growth of the granular culture, M12-4, on amino acid medium increased the number of lateral hyphae which formed along the length of most of the main hyphae (Pl. 1, fig. 5). The number of lateral hyphae did not increase when the fungus was grown on amino acid medium without methionine or on casein medium (Pl. 1, fig. 6).

Response of different species to methionine. Many granular and pleomorphic cultures were screened to determine their morphological response to amino acid medium. The New York Botanical Garden collection of granular and various pleomorphic cultures of *Trichophyton mentagrophytes*, when tested on amino acid medium (Table 1), could be grouped as follows in terms of response to methionine: (1) granular surface, pigmented reverse, positive morphological response; (2) typical fluffy pleomorph surface, white reverse, no response. A few atypical pleomorphs responded to methionine; they differed in having surface mycelium that was not fluffy, but was fuzzy or appressed with some pigment on the reverse.

Table 1. *Response of several species of dermatophyte fungi to methionine in an amino acid medium*

Organism	Source	Appearance on Neopeptone medium: Surface	Appearance on Neopeptone medium: Pigmentation on reverse side of colony	Morphological response to methionine. No. positive/no. tested
Trichophyton mentagrophytes	NYBG*	Granular	+	14/14
T. mentagrophytes	NYBG	Fluffy	−	0/10
T. mentagrophytes	NYBG	Fluffy	+	0/3
T. mentagrophytes	NYBG	Appressed	−	0/1
T. mentagrophytes	NYBG	Appressed	+	4/12
T. mentagrophytes	CDC†	Granular	+	7/8
T. mentagrophytes	CDC	Fluffy	−	0/2
T. mentagrophytes	AECM‡	Granular	+	1/1
T. rubrum	AECM	Granular	+	1/1
Microsporum audouinii	AECM	Granular	+	0/1
M. canis	AECM	Granular	+	0/1
M. gypseum	AECM	Granular	+	0/1
Epidermophyton floccosum	Brooklyn§	Granular	+	0/1

* New York Botanical Garden. † Communicable Disease Centre. ‡ Albert Einstein College. of Medicine. § Downstate Medical Centre.

Granular cultures of *Trichophyton mentagrophytes* obtained from other collections also responded to methionine. Pleomorphs, isolated from two of the Communicable Disease Centre cultures of *T. mentagrophytes* which responded to methionine, no longer showed the morphological modification of growth (Table 1). Species of Microsporum and Epidermophyton were tested on amino acid medium, but gave no positive response to methionine.

In addition to data on the response to methionine by the large number of stock Trichophyton cultures (Table 1), further tests were made on 112 new isolates derived from pleomorphic patches which formed on the surface of a granular culture of *Trichophyton mentagrophytes* (M12-4). Again, typical pleomorphs did

not respond to methionine, but a few, classified as atypical, did show a positive response, although most atypical pleomorphs did not respond.

Mechanism of action of methionine. Granular cultures responded whether methionine was added to the 19 L-amino acid medium before or after autoclaving, indicating the inducing agent was methionine and not a breakdown product. There was no effect when methionine (0·46 mM) was the sole source of nitrogen and, interestingly, there was very little methionine effect when methionine at 0·46 mM was added to casein medium. A positive morphological response to amino acid medium containing methionine depended neither on the type nor concentration of the sugar used as a source of carbon.

Table 2. *Effect of adding various compounds to an amino acid medium containing methionine on the morphological response of a granular culture of* Trichophyton mentagrophytes (*M* 12-4)

Compound added	Concentration (mM)	Effect of compound on methionine effect	
		Reverse	Increase
Thiourea	0·11	No	No
	0·23	Partial	No
	0·46	Complete	No
	1·4	Complete	No
S-Methyl-L-cysteine	0·46	No	No
	1·4	Partial	No
DL-Methionine sulfone	0·46	No	No
	1·4	Complete	No
DL-Methionine sulfoxide	0·46	No	No
	1·4	Partial	No
	2·3	Complete	No
Methionine, hydroxy analogue	0·46	No	No
	1·4	Partial	No

Intensity of the methionine effect depended on its concentration in the 19 L-amino acid medium; at 0·23 mM, the response was diminished as compared with that with 0·46 mM; when methionine was increased two- or threefold, the morphological response was greatly intensified. From a series of experiments it was concluded that a particular balance as well as concentration of amino acids, including methionine, was required to cause the methionine effect. There was a morphological response to methionine when the formula for hydrolysed casein (Dr W. J. Robbins, personal communication) or for casein (McMeekin, 1954) was used. When all 19 L-amino acids were supplied at 1·0 mM each, the methionine effect was diminished.

Several homologues and analogues of methionine were tested to determine whether any could mimic the methionine effect. Only D-methionine could replace the L-form but the effective concentration was three times that of the L-form. Among reducing compounds such as ascorbic acid, L-cysteine and thiourea, only the latter (Table 2) modified the methionine effect. S-methyl-L-cysteine (the lower homologue of methionine), DL-methionine sulphone, DL-methionine sulphoxide and methionine hydroxy analogue (α-hydroxy-γ-methylmercaptobutyric acid) annulled the methionine effect (Table 2). Incubation at 36° instead of 26°, or raising

the pH above 6·0 also annulled the methionine effect. Ethionine, homocystine, methylmethionine sulphonium chloride, methylmethionine sulphonium iodide, L-canavanine, 2,4-dinitrophenol, tryptazan, sodium malonate, sodium fluoride, sodium arsenite, chloramphenicol, or betaine HCl did not modify the methionine effect.

DISCUSSION

The pattern of growth response of *Trichophyton mentagrophytes* to the 19 L-amino acid medium has been used successfully as a scoring procedure in classifying morphologically typical or atypical pleomorphic isolates as being metabolically either granular or pleomorph. Although the mechanism whereby methionine exerts a morphological effect on granular cultures of *T. mentagrophytes* is yet unknown, this does not detract from its utility in revealing a metabolic difference between the normal granular and the pleomorphic cultures. This may mean that the working definition of pleomorphism in Trichophyton should now include the methionine effect.

Amino acids are known to play a role in cell division in lower organisms. In the fungus *Ustilago sphaerogena* (Spoerl, Saracheck & Smith, 1957) and the alga *Chlorella vulgaris* (Shrift, 1960) methionine specifically affected division processes. These effects were associated with one amino acid, while the change in morphology of the granular culture of *Trichophyton mentagrophytes* resulted from an interaction between methionine and one or more of the amino acids in the medium. Nevertheless, an effect on division processes was part of the morphological response in *T. mentagrophytes* since the number of lateral hyphae was increased when the fungus was grown on amino acid medium containing methionine.

The term 'paramorphogenic response' was coined by Tatum, Barrett & Cutter (1949) to describe the environmentally induced qualitative change in growth habit which occurred when Neurospora and Syncephalastrum were grown on media containing such chemicals as L-sorbose or sodium deoxycholate. The effect of methionine on *Trichophyton mentagrophytes* would seem to fit such a definition; however, the term may not be applicable here since the morphological response seems to be restricted to granular cultures of Trichophyton.

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EXPLANATION OF PLATE

Fig. 1. *Trichophyton mentagrophytes*, granular culture M12-4, on amino acid medium containing methionine; incubation for 17 days at 26°.

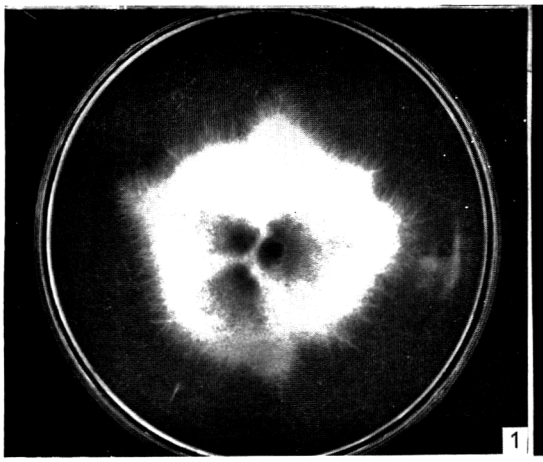
Fig. 2. Granular culture M12-4 on amino acid medium without methionine; incubation for 14 days at 26°.

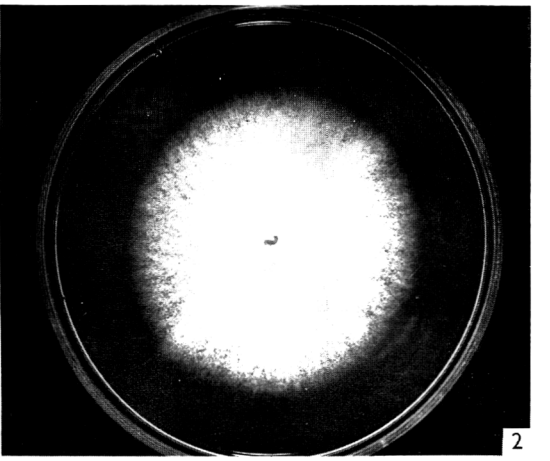
Fig. 3. *T. mentagrophytes*, pleomorphic culture T16-12, on amino acid medium containing methionine; incubation for 14 days at 26°.

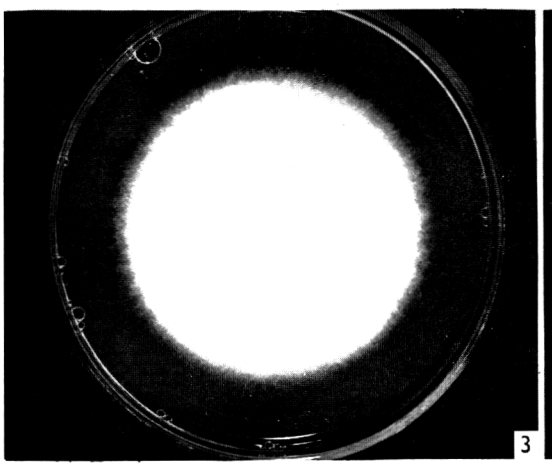
Fig. 4. Pleomorphic culture T16-12 on amino acid medium without methionine; incubation for 14 days at 26°.

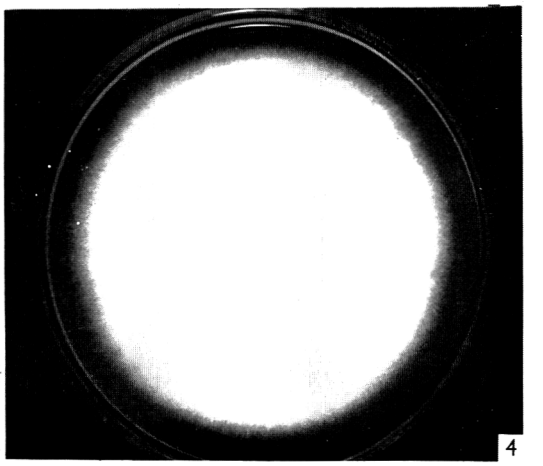
Fig. 5. Granular culture M12-4 on amino acid medium containing methionine.

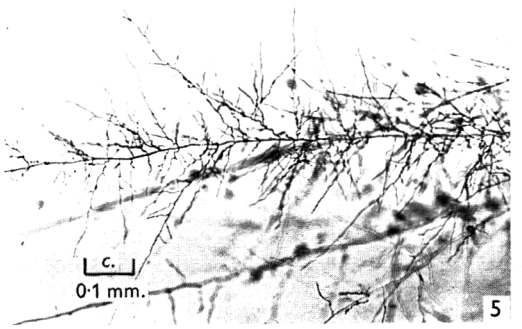
Fig. 6. Granular culture M12-4 on casein medium.

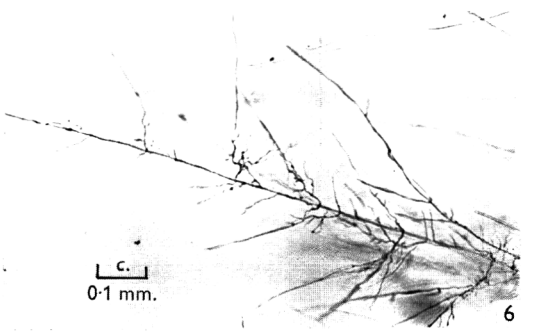












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The Effect of Heat on the Ability of a Host Strain to Support the Growth of a Staphylococcus Phage

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SUMMARY

A group of epidemiologically related strains of *Staphylococcus aureus*, which were untypable by standard methods, became sensitive to typing phage 187 after the inocula had been held at 56° for 2 min. The strains were found to be lysogenic. Evidence is presented to show that the phage carried, phage 5504′, was exerting interference against phage 187 which could be partially overcome by heat treatment. Phage 187 grown in heated cocci was found to have undergone a host-induced modification which rendered it capable of lysing unheated lysogenic organisms. Active multiplication of the carried phage, phage 5504′, appeared to be necessary for the multiplication of phage 187. Ultraviolet irradiation also increased the sensitivity of the strain to phage 187, but to a lesser degree than heat treatment. It is suggested that the effect of heating is to induce active multiplication of phage 5504′ in the lysogenic culture, possibly by destroying a cytoplasmic substance responsible for maintaining the carried phage in its prophage state.

INTRODUCTION

A collection of 14 epidemiologically related coagulase-positive staphylococci was received for bacteriophage typing from Professor Monaci of the Institute of Hygiene, The University of Parma, Italy, in the early part of 1961. These strains, which had been isolated from patients suffering from post-influenzal pneumonia in an old-people's home, were described by Muzzetto *et al.* (1961). They were not typable with the basic set of phages at routine test dilution (rtd) or at 1000 × rtd. Epidemics due to completely untypable strains of staphylococci are rare in our experience, and an extensive search was made amongst the experimental phages maintained in this laboratory in an attempt to find one which would lyse the strains or could be adapted for this purpose. No such phage was found. A report on the effect of heat on the typing pattern of strains of staphylococci by Ma & Mandle (1961) led us to repeat the typing of these strains after heating the inocula for 2 min. at 56°. After this treatment all strains were lysed by phage 187 at 1000 rtd. Phage 187 was originally designated phage 735A by Wahl & Fouace (1954) and is of interest because it is serologically distinct from all known staphylococcal phages, and strains lysed by this phage have never been found to be lysed by any other phage. Experiments to be described showed that the resistance of these strains to phage 187 was not due to failure of the unheated inocula to absorb the phage, but to interference exerted by a prophage carried by the strains. Heating appeared to overcome this

interference sufficiently to allow a limited multiplication of phage 187. The phage which grew on such heated inocula was found to be a host-induced modification of phage 187 which was able to multiply in the unheated strain. This phage will be referred to subsequently as modified phage 187 or 187*m*.

METHODS

Strains of Staphylococcus aureus *used.* (1) 14 strains of *S. aureus* from Professor Monaci. These strains behaved identically in that they became sensitive to phage 187 only after heating, and all carried an apparently identical temperate phage. The majority of experiments were carried out with one of them, strain 5504. (2) The propagating strain (PS) of phage 187, referred to as PS 187 and two lysogenic derivatives of this strain which will be described in the text.

Phage preparations. Phage 187 was grown on its propagating strain PS 187 by the agar layer method (Swanstrom & Adams, 1951). Phage 5504′, the phage carried by strain 5504, was originally prepared by filtering a broth culture of strain 5504, and subsequently by propagation of the phage on PS 187. Phage 187*m*, the host-range modification of phage 187 resulting from growth in heated cocci of strain 5504, was first prepared by isolating the plaques produced on a lawn arising from a heat-treated inoculum of strain 5504. Later preparations were made by propagating the phage in unheated cocci of strain 5504. All preparations of this phage contained some particles of phage 5504′ released by the lysogenic propagating strain but the proportion of phage 5504′ in preparations of phage 187*m* did not exceed 1 in 10^3 particles.

Phage antisera. Antisera against phages 187 and 5504′ were prepared in rabbits and tested in the usual way (Adams, 1950).

Heat treatment of staphylococcal strains. The most consistent results were produced by washing off the growth of an 18 hr. agar slope of the organism with 0·5 ml. broth to obtain a heavy suspension; 0·06 ml. of this suspension was added to 2 ml. nutrient broth previously warmed to 56°; this temperature was maintained for 2 min., after which the tubes were rapidly cooled and the suspension used to flood plates.

Tests for lysogenicity. Strains were tested for lysogenicity by spotting the supernatant fluid of a 4 hr. broth culture of the organism on indicator strains. The carried phage was given the same number as the strain from which it was derived, with the prime sign to distinguish it from the strain, i.e. phage 5504′ is the phage derived from strain 5504, the convention first proposed by Anderson & Felix (1953).

Test for non-lysogenic mutants. The lysogenic culture, in a dilution high enough to yield single colonies, was spread on agar plates. After overnight incubation at 37° these plates were replicated to plates previously flooded with a sensitive indicator strain (Lederberg & Lederberg, 1952). After incubation at 30° overnight each lysogenic colony on the replica plate was surrounded by a zone of lysis.

Lysogenization. Strains were lysogenized by spotting a sterile filtrate of the phage on to a lawn of organisms. The plates were incubated overnight at 30°, after which the secondary growth in the area of lysis was plated on an agar plate and, after incubation, colonies were tested for lysogenicity. A strain was considered to be lysogenic when it could be shown to be resistant to the lysogenizing phage, and to

carry that phage. The lysogenized strains are described in the usual way, i.e. PS 187(5504′) indicates PS 187 lysogenized by the phage from strain 5504 and PS 187(187) indicates PS 187 lysogenized by phage 187.

Efficiency of plating. The efficiency of plating (eop) of each of the three phages described in this paper is a measure of its titre on a given strain relative to its titre on PS 187 where in every case the titre was maximum or eop = 1.

RESULTS

All these epidemiologically related strains from Professor Monaci were resistant to the phages maintained in the Staphylococcus Reference Laboratory, even when tested at concentrations 10–100 times stronger than those used in routine techniques. After the inocula had been held at 56° for 2 min. all strains were lysed by phage 187 at 1000 rtd. A preparation of phage 187 containing 5×10^9 particles/ml., when titrated on PS 187, was tested on heated and unheated suspensions of strain 5504. A few small turbid plaques could be seen in the area where undiluted phage had been applied to the unheated cocci, representing a titre of about $2{\cdot}5 \times 10^2$/ml. The titre on the heated cocci was $2{\cdot}5 \times 10^5$. Heating had therefore increased the sensitivity of the strain to the phage a thousand fold and the eop of phage 187 was about 10^{-4} on the heated cocci and 10^{-7} on the unheated cocci. Sensitivity to phage 187 was gradually lost on subculture so that after 5 daily subcultures in broth the strain was again untypable.

The fact that heat treatment altered the cocci rather than the suspending medium was demonstrated when heated and unheated broth cultures were spun and their supernatant fluids exchanged. The heated cocci suspended in unheated supernatant fluid were sensitive to phage 187 while the unheated cocci in heated supernatant fluid remained insusceptible. The explanation of this phenomenon suggested by Ma & Mandle (1961) was failure on the part of the strains to absorb the phage. However, adsorption of phage 187 to unheated cocci of strain 5504 was as rapid as to heated cocci and more rapid than its absorption to PS 187.

Host-induced modification of phage 187 *resulting from growth in heated cocci*

Phage 187 had an eop of about 10^{-4} on heated cocci of strain 5504. This low efficiency suggested either that these plaques arose from mutant particles present in the stocks of phage 187, or from particles of phage 187 which had undergone host-induced modification. When the phage grown in heated cocci was examined it was found to be capable of lysing heated and unheated cocci with an equal eop of about 10^{-1}. It reverted to its original host range when subsequently propagated on PS 187 and lysed unheated cocci with an eop of 10^{-7} and heated cocci with an eop of 10^{-4}. These facts ruled out the possibility that phage 187 propagated on heated cocci of strain 5504 (phage 187*m*) represented a host-range mutant of phage 187 and indicated that host-induced modification was responsible for the alteration of host range. This phenomenon was fully described by Luria (1953) and is known to occur in *Salmonella typhi* (Anderson & Felix, 1952), *Escherichia coli* (Luria & Human, 1952; Bertani & Weigle, 1953) and in staphylococci (Ralston & Krueger, 1952).

Lysogenicity of the strains

PS 187 was tested for lysogenicity on a number of indicator strains and was found to carry a phage, phage 187′, which was serologically identical with typing phage 187. However, strain 5504 and the other epidemiologically related strains were completely resistant to this phage both before and after heat treatment, and the existence of phage 187′ did not appear to have any bearing on the phenomenon under investigation.

Strain 5504 and the other strains from this outbreak, on the other hand, were found to carry a phage, 5504′, which lysed PS 187 and all 25 other staphylococcal strains of phage type 187 which were tested. Phage 5504′ did not lyse any other indicator strain tested. This specificity suggested that it might be closely related to phage 187. However, 5504′ was not neutralized by antiserum prepared against phage 187 and did not belong to any of the known serological groups.

Antiserum was prepared against phage 5504′. This serum, and antiserum prepared against phage 187, were tested against the three phages, 187, 187*m* and 5504′. Antiserum against phage 187, at a dilution of 1/1000, neutralized about 90% of both phages 187 and 187*m* in 15 min; at a dilution of 1/100 it did not neutralize phage 5504′. 5504′ antiserum, at a dilution of 1/1000, neutralized 90% of phage 5504′ in 15 min., and at a dilution of 1/100 neutralized about 50% of both phages 187 and 187*m*. Since preparations of phage 5504′ used for immunization were made by propagating the phage on PS 187 (which is itself lysogenic for phage 187′), all such preparations will contain some phage 187′ as a contaminant, and antisera prepared against phage 5504′ will necessarily contain antibodies to phage 187′ and to the serologically identical phage 187. It seems most likely therefore that the cross-reaction observed when phage 187 was exposed to antiserum against phage 5504′ was due to these antibodies and that the two phages, 187 and 5504′, are serologically distinct.

Table 1. *Host ranges of the three phages*, 187, 187*m* *and* 5504′

Host range; strains of staphylococci	Phage		
	187	187*m*	5504′
	Efficiency of plating*		
PS 187	1	1	1
5504 unheated	10^{-7}	10^{-1}	—
5504 heated	10^{-4}	10^{-1}	—
PS 187(187) unheated	—	—	1
PS 187(187) heated	—	—	1
PS 187(5504′) unheated	10^{-7}	10^{-1}	—
PS 187(5504′) heated	10^{-4}	10^{-1}	—

* Efficiency of plating on strain PS 187 as 1.

PS 187 lysogenized with phage 5504′ (= PS 187(5504′)) was insensitive to phage 187; however, after heating, it was lysed by phage 187 with an eop of 10^{-4}. Phage 187 grown in these heated cocci was capable of lysing unheated PS 187(5504′) and unheated strain 5504 cocci. This strain therefore behaved in the same way as strain 5504, and carriage of phage 5504′ appeared to be responsible for the initial untypability of the strain.

Although carriage of phage 5504′ by PS 187 rendered the strain resistant to both phage 5504′ and phage 187, the reverse was not true. When PS 187 was lysogenized with phage 187 it became immune to phages 187 and 187*m* but remained fully sensitive to phage 5504′. Resistance to phage 187 in strains carring phage 5504′ resembled prophage interference as defined by Bertani (1958). The host ranges of the three phages, 187, 187*m* and 5504′ are summarized in Table 1.

The results so far reported suggested that carriage of phage 5504′ was exerting interference against phage 187 and that this interference was diminished by heat. It seemed possible that heating inactivated prophage 5504′ in a proportion of cocci, thus allowing multiplication of phage 187. If this were so it should have been possible to demonstrate non-lysogenic cocci amongst the survivors after heating. Repeated attempts to do this were unsuccessful. Furthermore, although loss of prophage seemed a possible explanation for the results obtained with strain 5504, it could not explain the results with PS 187(5504′), since the phage 187 produced in these cocci was indistinguishable from phage 187*m* in host range. If the cocci producing 187*m* in the PS 187(5504′) culture were non-lysogenic, phage 187 should not have undergone any modification, i.e. the phage produced should have been the same as phage 187 grown in PS 187. The presence of phage 5504′ in the productive cocci therefore appeared to be essential for the modification of phage 187. It was concluded that the effect of heating was not to inactivate prophage 5504′.

The inducing effect of heat treatment

Since loss of prophage 5504′ in strains 5504 or PS 187(5504′) could not explain the increased sensitivity of the strains to phage 187, the possibility that heat treatment induced the active multiplication of phage 5504′ in the lysogenic cocci was investigated. A broth suspension of strain 5504 was divided into two portions, one of which was held at 56° for 2 min. and rapidly cooled. Both suspensions were then diluted 1/100 in fresh broth and incubated at 37° for 6 hr. At 2-hourly intervals samples were removed and assayed for viable cocci and for free particles of phage 5504′ in the supernatant fluids after centrifugation. At the same time the undiluted suspensions were tested for sensitivity to phage 187. Figure 1 shows the result of this experiment. It can be seen that the viable count fell by 76% as a result of heat treatment, while the free phage count fell by 97%. During the 6 hr. incubation period the viable count in the unheated culture rose logarithmically after an initial lag period. The concentration of free phage rose from 1×10^5/ml. to 5×10^6/ml. during the 6 hr. period, with an average coccus:phage ratio of 60:1.

In the heated suspension the viable count remained approximately stationary. The free phage concentration, however, increased from 3×10^3 to $1{\cdot}3 \times 10^6$ so that after 6 hr. the coccus:phage ratio was about 1:1. This represented a 450-fold increase in free phage, whereas the increase in the unheated control culture was only 50-fold. The amount of free phage produced by the heated cocci compared with that produced by unheated cocci provided evidence that heat treatment was inducing active multiplication of the temperate phage in the lysogenic culture.

Sensitivity to phage 187 was about 1000 times greater in the heated suspensions than in the unheated ones, but in both cases sensitivity increased during the 6 hr. period. The eop on the heated cocci increased from 5×10^{-5} to 2×10^{-4} but the eop on the unheated cocci, although only 5×10^{-9} at the start of the experiment,

was 4×10^{-7} after incubation for 6 hr. It appeared therefore that ageing of the culture alone was responsible for some increase in sensitivity of the cocci to phage 187. This was confirmed by a further experiment in which a broth culture of strain 5504 was held at 37° for 30 hr. and tested for sensitivity to phage 187 after incubation for 6, 24 and 30 hr. The eop of the phage at these times was, respectively, 3×10^{-6}, 4×10^{-6} and $1{\cdot}4 \times 10^{-5}$.

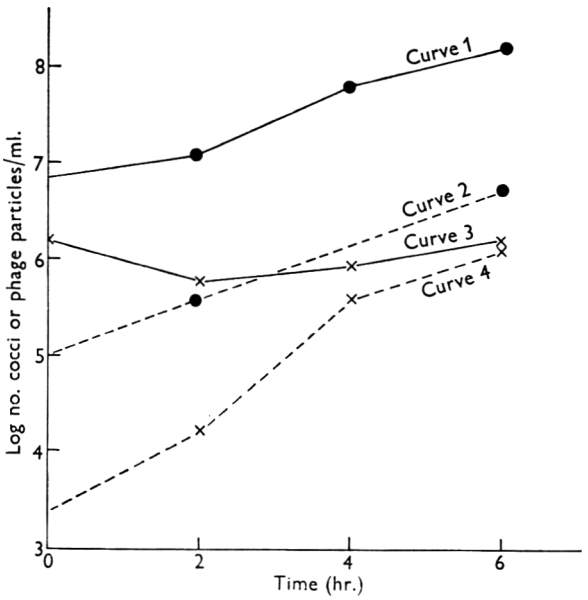

Fig. 1. Growth of organism and production of free phage in strain 5504 after no heat treatment and after exposure to 56° for 2 min. Curve 1 = count of cocci in unheated suspension; curve 2 = free phage produced by the unheated culture; curve 3 = count of cocci in heated suspension; curve 4 = free phage produced by heated suspension.

Production of phage 187 m in doubly infected cocci

Since heat treatment had an inducing effect on the lysogenic strain and since such treatment was known to increase the proportion of cocci which phage 187 could infect, leading to production of phage 187 *m*, it seemed probable that these two effects were correlated, i.e. that active multiplication of phage 5504′ was necessary for the production of phage 187 *m*. This seemed likely in view of Christensen's results with *Shigella dysenteriae* strain Sh lysogenic for phage P 1 (Christensen, 1961). In this strain phage T 1 undergoes a host-controlled modification. Christensen's results indicated that lysogenic organisms which produced modified phage T 1 were those in which active multiplication of phage P 1 was taking place, and that some event dependent upon vegetative multiplication of the carried phage was responsible for the modification of the infecting phage. Experiments were made to see whether the same situation existed here. PS 187 and 187(5504′) were used, since all the evidence suggested that PS 187(5504′) behaved in exactly the same way as strain 5504. Each of the two strains was infected first with phage 5504′ and then with phage 187. Titrations were made during the latent period to count

Table 2. *Multiplication of phages* 187, 187*m* *and* 5504′ *in cocci of staphylococcal strains* PS 187 *and* PS 187(5504′) *singly or doubly infected with phages* 5504′ *and* 187

Tube no.	Contents: Staphylococcus Strain	Contents: Staphylococcus Cocci/ml.	Contents: Phage/ml. 5504′	Contents: Phage/ml. 187	Infective centres at 20–25 min. titrated on: Strain PS 187 (*a*)	Strain 187(187) (*b*)	Strain 187(5504′) (*c*)	Yield of phage at 2 hr.: Phage 187	Phage 5504′	Phage 187
1	PS 187	$1{\cdot}3 \times 10^8$	4×10^8	—	$9{\cdot}5 \times 10^7$	1×10^8	—	—	$1{\cdot}1 \times 10^{10}$	—
2	PS 187	$1{\cdot}3 \times 10^8$	—	$6{\cdot}4 \times 10^8$	7×10^7	—	—	$1{\cdot}1 \times 10^{10}$	—	—
3	PS 187	$1{\cdot}3 \times 10^8$	4×10^8	$6{\cdot}4 \times 10^8$	$1{\cdot}2 \times 10^8$	$1{\cdot}05 \times 10^8$	$3{\cdot}5 \times 10^6$	$1{\cdot}7 \times 10^9$	7×10^9	1×10^7
4	187(5504′)	$2{\cdot}4 \times 10^8$	4×10^8	$6{\cdot}4 \times 10^8$	$3{\cdot}9 \times 10^7$	$3{\cdot}5 \times 10^7$	$1{\cdot}5 \times 10^3$	$< 5 \times 10^5$	5×10^5	3×10^3

Log phase broth cultures of PS 187 (tube 3) and 187(5504′) (tube 4) were mixed with phage 5504′ and after 5 min. phage 187 was added. After a furth 5 min. the infected cocci were diluted into broth containing antisera against each of the phages, 5504′ and 187. 10 min. were allowed for serum inactivati of unadsorbed phage and the cocci were further diluted in broth held at 37°. Titrations to count infective centres were made at 20–25 min. on three indica strains, PS 187, 187(5504′) and 187(187). The tubes were held at 37° for a total of 80 min. when lysis was complete. They were then each divided into t parts, one part being treated with antiserum against phage 5504′ and the other with antiserum against phage 187 and 187*m*. After allowing 10 min. inactivation of the specific phage the contents were titrated on the same three indicator strains to determine the yield of each of the three phages, 1 187*m* and 5504′. Tubes 1 and 2 which represent control tubes in which PS 187 was singly infected with phage 5504′ (tube 1) and phage 187 (tube 2) w included to measure the rate of infection of the strain with each of these phages.

infective centres and again, when lysis was complete, to determine the yield of the three phages, 187, 187*m* and 5504′.

Three indicator strains were used, namely PS 187, PS 187(187) and PS 187(5504′). The sensitivities of these strains are shown in Table 1. PS 187 was fully sensitive to phage 187, 187*m* and 5504′ and all infective centres and free phage particles produced a plaque on this strain; PS 187(5504′) was sensitive only to phage 187*m*; PS 187(187) was sensitive only to phage 5504′. The difference between the counts on PS 187 and PS 187(187) represented cocci which released only phage 187. It was not possible to distinguish cocci which released only phage 5504′ from cocci which released phage 187 and phage 5504′, since no strain sensitive only to phage 187 was available. Control tubes were therefore included containing PS 187 infected separately with each of the two phages, 187 and 5504′, and the number of doubly infected cocci was calculated from the figures obtained in these tubes, assuming that a coccus infected with phage 5504′ was as susceptible to infection with phage 187 as was an uninfected coccus. Experiments had shown that infection of PS 187 with phage 5504′ did not affect the adsorption rate of phage 187.

It was expected that if active multiplication of phage 5504′ were essential for the production of phage 187*m*, the amount of modified phage would be greatest where PS 187 was first infected with phage 5504′ and subsequently with phage 187, since, in these cocci, phage 5504′ would be actively multiplying. Where PS 187(5504′) was exposed to phage 5504′ and then to phage 187, only the occasional cocci which underwent spontaneous induction would be expected to produce phage 187*m*, unless superinfection of the strain with phage 5504′ had the effect of inducing active multiplication of the carried phage. The results of this experiment, shown in Table 2, can be summarized as follows. Tube 1: in PS 187 infected with phage 5504′ the proportion of cocci infected ($1-e^{-x}$) was 0·75, and the mean burst size (mbs) was 118 phage particles. Tube 2: in PS 187 infected with phage 187 the proportion of cocci infected ($1-e^{-y}$) was 0·54 and the mbs was 157 phage particles. Tube 3: with PS 187 first infected with phage 5504′ and then with phage 187 the platings at 20–25 min. allowed the following figures to be deduced. Total infective centres = $1{\cdot}2\times10^{8}$ (column *a*): cocci releasing phage 5504′ = $1{\cdot}05\times10^{8}$ (column *b*); doubly infected cocci, calculated from the Poisson formula = $5{\cdot}2\times10^{7}$; cocci releasing only phage 187 (column *a* − column *b*) = $1{\cdot}5\times10^{7}$; cocci releasing only phage 5504′ ($1{\cdot}05\times10^{8}-5{\cdot}2\times10^{7}$) = $5{\cdot}3\times10^{7}$; cocci releasing phage 187*m* = $3{\cdot}5\times10^{6}$ (column *c*) or 6·7 % of the doubly infected cocci. It was not possible to determine from the figures obtained from the 20–25 min. platings whether doubly infected cocci released phage 187 as well as phage 5504′, since an indicator strain sensitive to phage 187 and resistant to phage 5504′ was not available. However, the yield of phage 187 in tube 3 at 2 hr. was only $1{\cdot}7\times10^{9}$. On the assumption that all cocci infected with phage 187, regardless of whether or not they were also infected with phage 5504′, released some phage 187, the mbs for phage 187 in tube 3 was only 25 particles. The mbs for phage 187 in tube 2 was 157 particles. If the cocci in tube 3 which released only phage 187 ($1{\cdot}5\times10^{7}$) released a normal burst, these cocci alone could account for all the phage 187 produced in this tube. It is possible that doubly infected cocci released a small burst of phage 187, since the yield of phage 187*m* by those cocci that released any phage 187*m* was only three particles per coccus. A similar yield of unmodified phage 187 would not have been detected. The mbs

for phage 5504′ in tube 3 was 66 particles as compared with 113 in tube 1. This suggested that adsorption of phage 187 to cocci infected with phage 5504′ had a 'depressor effect' on the multiplication of phage 5504′ (Delbrück, 1945). Tube 4: PS 187(5504′) infected first with phage 5504′ and then with phage 187. The figures obtained showed no detectable multiplication of phage 187. Phage 5504′ cannot multiply in PS 187(5504′) because of the immunity provided by the carried phage. The apparent increase in phage 5504′ in this tube could be shown to be due to spontaneous lysis, since an identical culture to which no phage was added produced a similar number of plaques when plated on PS 187 and 187(187). Phage 187*m* was produced by $1{\cdot}5 \times 10^3$ cocci as compared with $3{\cdot}5 \times 10^6$ in tube 3. When PS 187(5504′) was infected only with phage 187, without superinfection with phage 5504′, the number of cocci producing phage 187*m* was of the same order.

In general, these results are like those described by Christensen (1961) for a Shigella system, the only difference being that, in his system, phage P 1, which exerted interference against phage T 1 when in the prophage state, did not do so when it was in the vegetative state, so that organisms doubly infected with phage P 1 and T 1 produced unmodified, as well as modified, phage T 1. The results presented here and the results of similar experiments with phages 5504′ and 187 provided evidence that phage 5504′ exerts interference against phage 187, both when the former is in the prophage state and when it is multiplying vegetatively. In both cases, however, the amount of modified phage produced was considerably higher when a non-lysogenic culture was doubly infected with the two phages than when the corresponding lysogenic strain was so treated. The small amount of 187*m* produced by infecting PS 187(5504′) with the two phages probably occurred in cocci undergoing spontaneous lysis. We conclude, therefore, that actively multiplying phage 5504′ is essential for the production of phage 187*m*.

Since the amount of 187*m* produced in strains lysogenic for phage 5504′ was dependent on the presence of actively multiplying phage 5504′ and since heat treatment induced active multiplication of the carried phage, it is concluded that it is the inducing action of heat which increases the sensitivity of strain 5504 to phage 187.

The effect of ultraviolet irradiation

Ultraviolet (u.v.) irradiation is known to have an inducing effect on many lysogenic bacteria (Lwoff, Siminovitch & Kjeldgaard, 1950). Strain 5504 was exposed for varying lengths of time to radiation of wave length 2537 Å. from a 15 W. low-pressure germicidal lamp at 17 cm. from the u.v. source. We did not observe any induction of the cultures but the treatment increased the sensitivity of the strain to phage 187. Agar plates, flooded with a broth culture of strain 5504 were irradiated for 10 sec. and phage 187 was then titrated on them. The eop of the phage was $66{\cdot}0 \times 10^{-7}$ as opposed to $3{\cdot}3 \times 10^{-7}$ on the unirradiated controls. A similar effect of u.v. irradiation was described by Bertani & Weigle (1953) on *E. coli* strain S. On this strain phage λC plates with a very low eop and the plaques that are produced represent a host-induced modification of the phage. These authors showed that irradiation of strain S increased the eop of phage λC on the strain, and suggested that irradiation changed the physiological condition of the organisms.

DISCUSSION

The experiments described show that the untypability of the collection of strains received from Professor Monaci was due to interference exerted by a carried phage, of which phage 5504′ has been selected as the representative, against the typing phage, 187. This interference was partly overcome by heating the inocula at 56° for 2 min., which allowed multiplication of phage 187 in a small proportion of the cocci. It was first considered that the action of heat might be to eliminate the carried phage in a few cocci and thus to overcome the interference. However, this possibility was ruled out for two reasons: (i) no non-lysogenic cocci were detected in heated cultures; (ii) phage 187 produced in heated cocci was modified in its host range in such a way that it was capable of lysing unheated cocci. This modified phage was produced not only in strain 5504 but also in PS 187 lysogenized with phage 5504′. In the latter strain any non-lysogenic cocci would be expected to produce unmodified phage 187. Since only modified phage 187 was produced in PS 187(5504′) after heating, the effect of heating could not have been to eliminate phage 5504′. The evidence suggested that active multiplication of the carried phage was essential for the production of a modified phage 187 (187*m*). In view of this, phage 187*m* might be expected to represent a genetic recombinant between phage 5504′ and 187. However, the phage 187*m* reverted to typical phage 187 after growth in PS 187, a finding consistent with its being an example of host-induced modification rather than a host-range mutant of phage 187, or a genetic recombinant of phage 5504′ and 187. Phage 187*m* differed from the original phage 187 only in its ability to grow in unheated cocci lysogenic for phage 5504′; it was identical serologically and in plaque morphology with phage 187.

Heat treatment of strains lysogenic for phage 5504′ had a slight inducing effect and it is suggested that this induction is responsible for the increased sensitivity of the strains to phage 187, since it would provide more cocci containing actively multiplying phage 5504′, and thus more cocci in which phage 187*m* could be produced. It is possible that induction by heat is brought about by destroying a cytoplasmic repressor substance which, it is suggested, is responsible for maintaining the stability of the prophage state in the strain (Jacob & Campbell, 1959). Treatment with u.v. radiation also increased the sensitivity of the strains to phage 187. Perhaps heat treatment and u.v. irradiation both produce their effects by destroying a cytoplasmic repressor substance.

The increase in the eop of phage 187 from 10^{-7} to 10^{-4} as a result of heat treatment was sufficiently great to enable these epidemiologically related strains to be recognized as being identical. Since 1000 rtd preparations of phages used in routine typing of staphylococci contain about 10^8 particles/ml., the strains were not lysed, and would not have been recognized as belonging to the phage type had they not been treated by heat. It is possible that, where a host-induced modification of the typing phages can take place, a similar effect may be observed on other strains of staphylococci, which would increase the strength of the reactions and thereby widen the spectrum of lysis. It is questionable, however, whether such an expansion of the lytic spectrum would have any practical value except in special cases such as the one described here, where a strain would otherwise be untypable. The effect of heating was investigated on two other strains of staphylococci. One

of these strains, previously not sensitive to phage 80, became sensitive after heating, and with the other strain the same effect was demonstrated with phage 71. In both cases the phages grown in the heated strains had been modified in that they now lysed unheated cocci of the same strain. This was not investigated further; the phenomenon does not appear to be uncommon.

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The Metabolism of Glycyl-L-leucine in *Escherichia coli*

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SUMMARY

The metabolism of glycyl-L-leucine in a leucine auxotroph of *Escherichia coli* strain K-12 was investigated with respect to the bacteriostatic effect of the peptide on growth, its hydrolysis by bacterial preparations, and its uptake by resting organisms. Growth inhibition by the peptide is relatively small if the organisms used as the inocula for the growth tests are taken from actively multiplying cultures (young organisms), but is marked if the inocula consist of organisms from cultures aged in an acid-producing, poorly buffered medium; ageing in a well-buffered or neutral medium does not produce peptide-sensitive organisms. Aged organisms that are very sensitive to the peptide readily take up the compound from the external medium, both in the absence and presence of chloramphenicol. Whole-cell preparations of these aged organisms do not effect the rapid hydrolysis of glycylleucine, but cell-free extracts have as much enzymic activity as cell-free extracts of young organisms. Whole-cell preparations of young organisms also have high enzymic activity. The available data suggest that the uptake of glycylleucine by young organisms is followed immediately by its hydrolysis, whereas the peptide taken up by aged (peptide-sensitive) organisms is hydrolysed only very slowly. The growth of inocula composed of organisms taken from yeast extract + peptone + agar slopes is markedly inhibited by glycylleucine. The hydrolytic activity of whole organisms taken from slopes is significantly less than that of young organisms, but disruption of slope-grown organisms to produce cell-free extracts does not lead to any significant increase in enzymic activity.

INTRODUCTION

Some years ago it was reported that a leucine-requiring auxotroph of *Escherichia coli* strain K-12 can utilize for growth a variety of di-, tri- and tetrapeptides containing L-leucine and glycine (Simmonds, Tatum & Fruton, 1947; Simmonds & Fruton, 1949). Although the final extent of growth of the auxotroph (strain 679-680) was the same in media containing free L-leucine or an equivalent concentration of peptide-bound leucine, the peptides exerted an inhibitory effect on the initiation of growth, which increased as the initial concentration of the peptide in the medium was increased (Simmonds & Fruton, 1949; Simmonds, Harris & Fruton, 1951). A similar bacteriostatic effect on the initiation of growth by leucine peptides was observed with the prototroph (strain K-12) and with a threonine auxotroph (strain 679) (Simmonds *et al.* 1951).

The bacteriostasis was evident when the leucine auxotroph was cultured either in the absence or the presence of exogenous L-leucine, so that the 'lag' time of the growth cycle always was determined by the initial concentration of inhibitory

peptide, whilst the final extent of growth depended upon the sum of the free + the peptide-bound leucine added to the medium. Of particular interest in relation to the peptide metabolism of bacteria was the observation that inocula composed of 'young organisms' (i.e. bacteria taken from a culture in a synthetic liquid medium prior to the completion of the growth cycle) showed less sensitivity to an inhibitory dipeptide (e.g. glycyl-L-leucine) than did 'slope organisms' harvested from 24 hr. slopes composed of agar, yeast extract and peptone; on the other hand, 'aged organisms' taken from the liquid medium 85–95 hr. after the culture had attained maximal growth were much more sensitive to the inhibitor.

These observations led to the conclusion that hydrolysis of the peptides is probably a required step in the utilization of peptide leucine for growth and also the mechanism for the reversal of the inhibition (Simmonds *et al.* 1951). It was suggested that the decreased inhibitory effect observed with inocula composed of young organisms might be due to a relatively high enzymic activity in the hydrolysis of the peptides (Simmons & Fruton, 1949). The experiments to be reported here were undertaken in an attempt to determine the validity of these hypotheses and, in particular, to study the effects of 'ageing' on the bacterial metabolism of leucine peptides such as glycyl-L-leucine.

METHODS

Organism. The leucine auxotroph used, strain 679-680, was the leucine- and threonine-requiring mutant of *Escherichia coli* strain K-12 described by Simmonds *et al.* (1947). Stock cultures were maintained on slopes of 'complete-agar': 0·5% (w/v) Difco yeast extract + 0·3% (w/v) Difco peptone + 1·5% (w/v) Difco agar.

Growth tests. The procedures followed were essentially those of Simmonds, Harris & Fruton (1951). The two basal media used were similar in composition to media A and C of Simmonds & Griffith (1962), and differ only in nitrogen sources and buffering power: medium A has NH_4^+ salts and 25 mM-phosphate, medium C has glycine and 100 mM-phosphate. In the present experiments, each basal medium also contained 0·8 mM-DL-threonine (to satisfy the requirement of strain 679-680 for that amino acid) and was supplemented either with L-leucine or with glycyl-L-leucine in the concentrations indicated for each experiment.

Inocula consisted of bacteria taken either from 24 hr. cultures on complete-agar slopes ('slope organisms'), from 24 hr. cultures in 'complete-broth' (the yeast extract + peptone mixture in water) or from cultures in one of the basal media supplemented with 240 μM-L-leucine. Organisms from slopes were suspended in a sterile aqueous solution of glucose (40 mg./ml.) at a concentration of *c.* 2×10^7 cells/ml. to give an inoculum suspension of optical density (i.e. absorbancy) in an Evelyn colorimeter (Minneapolis-Honeywell Regulator Co., Philadelphia, Pa.), filter no. 540. For inocula composed of organisms from liquid cultures, a portion of the culture was diluted with the sterile glucose solution to the desired optical density. Such inoculum suspensions, therefore, contained a small amount of culture fluid; at most this contaminating fluid was 0·3 ml./ml. of inoculum suspension. Samples of 'young organisms' were removed from the liquid cultures at the time of approximately half-maximal growth, i.e. *c.* 30 hr. after inoculation with slope organisms, and samples of 'aged organisms' at *c.* 100 hr. after attainment of maximal growth, i.e. about 140 hr. after inoculation.

Growth of cultures, which consisted of a final volume of 10 ml. of medium containing *c.* 10^7 cells (added as the inoculum suspensions described above), was measured by periodic optical density determinations (Evelyn colorimeter, filter no. 540). Cultures were incubated at 25° either statically or on a shaker, as specified for each experiment. The times required for cultures to reach half-maximal optical density ($t_{\frac{1}{2}}$) in the presence of each test compound were determined, and the extent of peptide inhibition, expressed as $\Delta t_{\frac{1}{2}}$, was estimated by subtracting $t_{\frac{1}{2}}$ for leucine from $t_{\frac{1}{2}}$ for the equimolar concentration of glycyl-L-leucine.

Hydrolysis experiments. Strain 679-680 organisms were grown at 25° either on slopes of complete agar for 24 hr., or in 500–1000 ml. of medium A containing 1 mM-L-leucine for the time required to produce young or aged organisms. The liquid cultures were incubated statically or on a shaker as specified for each experiment, and the bacteria were collected by centrifugation (2500 rev./min., room temperature), washed twice with 0·9% (w/v) NaCl solution and then resuspended in the saline solution. Organisms were removed from slopes on cotton swabs and suspended in 0·9% saline. The final saline suspensions contained an amount of fresh organisms equivalent to approximately 1 mg. dry weight/ml. For the preparation of cell-free extracts, the whole bacteria were frozen and crushed in a Hughes press. The crushed material was suspended in saline (the resulting suspension contained the equivalent of 1 mg. dry weight of whole bacteria/ml.), and centrifuged to remove cell debris. Lyophilized preparations also were made from frozen whole organisms.

Complete reaction mixtures (10 ml. final volume) consisted of glycyl-L-leucine (2 mM) and one of the various bacterial preparations in a solution (pH 7·1) containing Na_2SO_4, phosphate, $MgSO_4$, and 'trace elements' in the concentrations at which these salts are present in basal medium A. Thus, the hydrolysis reaction medium resembled the basal growth medium except for the omission of all inorganic nitrogen compounds, asparagine and glucose. Control mixtures lacking either the dipeptide or the bacterial preparations were included in each experiment. All reaction mixtures were incubated in tightly stoppered 10 ml. volumetric flasks in a water bath at 38° (other conditions as specified for each experiment). Samples (1 ml.) were removed at zero time, and at intervals thereafter, and placed in small test tubes which were heated in a boiling water bath for 10 min. and then centrifuged. Samples (0·1 ml.) of the deproteinized mixtures were analysed, in triplicate, for amino acid nitrogen by the photometric ninhydrin method to measure the extent of hydrolysis of the dipeptide (Schwartz & Engel, 1950; Meinhart & Simmonds, 1955). Equimolar concentrations of glycylleucine, leucine, and glycine have approximately equal colour values in this ninhydrin analysis. Standard curves were prepared with mixtures of dipeptide + glycine + leucine in the proportions expected for from 0 to 100% hydrolysis.

When NH_3 production was measured, 0·4 or 0·5 ml. of a deproteinized mixture was diluted with water to 10 ml. and treated with Nessler reagent (Seligson & Seligson, 1951); the dipeptide present in the sample does not interfere with the Nesslerization reaction.

For paper chromatographic examination of the deproteinized mixtures, 0·1 ml. samples were spotted on paper; the developing solvent (ascending) was *n*-butanol + pyridine + water (2:1:1, by vol.). R_F values were: leucine, 0·52; glycylleucine, 0·27;

glycine, 0·08. When glycyl-[^{14}C]L-leucine was the substrate, the areas on the paper chromatograms corresponding to the peptide and to leucine were eluted, and their ^{14}C-contents determined. The extent of hydrolysis was calculated from both the amount of labelled peptide that disappeared and the amount of labelled leucine that was formed.

Uptake experiments. The bacteria were grown on complete-agar slopes or in medium A supplemented with leucine as described in the preceding section. Young organisms were prepared in shaken cultures, aged organisms in static cultures. The organisms were collected by centrifugation in the cold, washed twice with cold distilled water to free them of contaminating threonine (and leucine), and resuspended in sufficient cold basal medium A (devoid of threonine and any leucine-containing compound) to provide a suspension of optical density 0·09–0·10 (equivalent to a wet-cell volume of about 0·5 μl., and a concentration of 100 μg. dry weight of bacteria, per ml.). This suspension was kept at 25° for 30 min. prior to the addition of leucine or glycylleucine. The subsequent procedure was that of Levine & Simmonds (1960) except for the following modifications: (*a*) all incubations were at 25°, and the mixtures containing aged organisms were incubated statically rather than on a shaker; (*b*) chloramphenicol was used at a concentration of 100 μg./ml. of medium; (*c*) 2 ml. samples of the test cultures were removed for collection of the organisms and ^{14}C-analysis by the 'Millipore method'. To calculate the mμmoles of compound in the organisms collected at any specified time, the observed radioactivity was corrected by subtraction of the radioactivity in the zero-time sample. Zero-time samples had 0·6–1·8 % of the ^{14}C-content of 2 ml. of media. In experiments with labelled compounds at a concentration of 100 μM, the maximal error of the values given (for the organisms collected from 2 ml. of culture) is estimated to be $\pm$0·05 mμmole; with labelled compounds at 50 μM, this error is $\pm$0·03 mμmole. The isotope content of whole organisms, expressed as mμmoles of labelled test compound in the organisms from a 2 ml. sample of culture, divided by the mM concentration of test compound in the 'uptake medium' is a measure of the extent to which the test compound is 'concentrated' by the bacterial cells.

Test compounds. Uniformly labelled [^{14}C]L-leucine (purchased from Nuclear-Chicago Corporation, Chicago, Ill.) and the glycyl-[^{14}C]L-leucine prepared (Vaughan & Osato, 1952) from it were diluted with the corresponding unlabelled compounds to the desired specific activities. Unlabelled amino acids and glycyl-L-leucine were gifts from Dr Joseph S. Fruton.

RESULTS

Growth tests. Experiments were carried out to determine whether the extent to which the growth of the leucine auxotroph is inhibited by glycylleucine depends on the conditions under which the growth tests are made as well as on the 'type' of organisms used as the inocula (Table 1). The results indicate that inocula composed of a given 'cell-type' prepared under a given set of conditions (e.g. slope organisms) show approximately the same relative growth responses to leucine and glycylleucine ($\Delta t_{\frac{1}{2}}$ values) whether these responses are measured during static or shaken incubation. That the more highly aerobic conditions produced by shaking does not influence the results of the growth tests is shown also by the experiment in which slope organisms tested under N_2 were found to behave just like slope organisms tested in air.

Table 1. *Effect of glycyl-L-leucine on the growth of the leucine auxotroph of* Escherichia coli K-12

Growth tests were carried out in the media indicated supplemented with L-leucine or glycyl-L-leucine at the initial concentrations of 80 and 240 μM. The precision of the measurements of $t_{\frac{1}{2}}$ and $\Delta t_{\frac{1}{2}}$ (defined in Methods) is indicated by the average deviation of the values found in separate experiments; the number of such separate experiments is shown in parentheses.

Inoculum preparation			Growth tests*							
			Static cultures				Shaken cultures			
			80 μM		240 μM		80 μM		240 μM	
Medium and incubation conditions	Cell type produced	Basal medium used	$t_{\frac{1}{2}}$ on leucine (hr.)	$\Delta t_{\frac{1}{2}}$ (hr.)	$t_{\frac{1}{2}}$ on leucine (hr.)	$\Delta t_{\frac{1}{2}}$ (hr.)	$t_{\frac{1}{2}}$ on leucine (hr.)	$\Delta t_{\frac{1}{2}}$ (hr.)	$t_{\frac{1}{2}}$ on leucine (hr.)	$\Delta t_{\frac{1}{2}}$ (hr.)
Complete-agar	Slope	A	33 ± 8 (2)	$+7 \pm 2$	$29 + 3$ (3)	$+33 \pm 3$	26 ± 3 (3)	$+8 \pm 3$	31 ± 2 (3)†	$+30 \pm 4$†
Complete-agar	Slope	C	—	—	41 (1)	+29	—	—	—	—
A; static	Young	A	15 ± 2 (3)	$+4 \pm 1$	16 ± 2 (3)	$+12 \pm 3$	—	—	16 (1)‡	+6‡
A; static	Aged	A	36 ± 3 (2)	$+23 \pm 8$	36 ± 2 (2)	$+42 \pm 1$	—	—	32 ± 4 (2)§	$+20 \pm 6$§
C; static	Aged‖	C	—	—	16 (1)	+4	—	—	—	—
Complete-broth; static	¶	A	11 (1)	+2	14 ± 3 (2)	$+11 \pm 3$	—	—	—	—
A; shaken	Young	A	—	—	—	—	14 ± 2 (2)	$+0 \pm 2$	14 ± 0 (2)†	$+6 \pm 1$†
A; shaken	Aged	—	—	—	—	—	17 ± 1 (3)	$+1 \pm 1$	20 ± 2 (3)†	$+3 \pm 1$†

* Maximal optical densities for all cultures in medium A supplemented with 80 and 240 μM-leucine or glycylleucine were 0·14–0·16 and 0·14–0·18, respectively, after static incubation, and 0·29–0·32 and 0·55–0·68, respectively, after shaken incubation. For static cultures in medium C with 240 μM-test compound, the maximal optical density was *c.* 0·32.

† The same values were obtained in an experiment with inocula prepared and subsequently tested under N_2 rather than air.

‡ Each 10 ml. of growth-test medium was supplemented with 0·15 ml. of cell-free fluid obtained by centrifugation and sterile-filtration of a statically aged culture; i.e. with the amount 'contaminating' the usual inoculum of aged organisms prepared in a static culture.

§ The same values were obtained with an inoculum of organisms aged in a static culture and freed of contaminating culture fluid by centrifugation.

‖ When the sample was removed to provide the inoculum for growth tests, the pH of the culture was 7·0 (cf. data for pH changes in medium A, Table 2).

¶ Samples were removed for use as inocula after 24 hr. incubation; this is *c.* 6 hr. after the attainment of maximal growth, which is characterized by an optical density of *c.* 0·29.

The conditions established by shaking or static incubation during the preparation of inocula have little significant effect on the extent to which the subsequent growth of young organisms is inhibited by the peptide. However, organisms aged in shaken cultures of medium A lack the high sensitivity to glycylleucine that is a characteristic property of cells aged in static cultures (cf. lines 8 and 4, Table 1).

Table 2. *Production of sensitivity to glycyl-L-leucine in the leucine auxotroph of* Escherichia coli K-12

Organisms from a complete-agar slope were inoculated into a series of tubes containing 10 ml. of medium A supplemented with 240 μM-L-leucine and into two tubes of this medium supplemented with 240 μM-glycyl-L-leucine. At each 'incubation time' shown, a sample was removed from one of the cultures growing on leucine to serve as the inoculum for a second growth test in the same types of media, and the pH of the remaining culture was measured. Values for $t_{\frac{1}{2}}$ and $\Delta t_{\frac{1}{2}}$ were determined as described in Methods.

	Inoculum preparation				Growth-test response		
Medium and incubation conditions	Incubation time (hr.)	Culture optical density	Culture pH*	Cell-type produced	Incubation conditions	$t_{\frac{1}{2}}$ on leucine (hr.)	$\Delta t_{\frac{1}{2}}$ (hr.)
Complete-agar	24	—	—	Slope	Static	34	+29
A; static	32	0·07	6·4	Young	Static	18	+9
	47	0·16	5·6	—	Static	17	+8
	102	0·14	5·3	—	Static	18	+6
	127	0·13	5·4	—	Static	26	+11
	153	0·15	5·5	Aged	Static	47	+31
Complete-agar	24	—	—	Slope	Shaken	22	+25
A; shaken	24	0·29	6·2	Young	Shaken	15	+6
	40	0·56	5·8	—	Shaken	16	+5
	98	0·59	6·4	—	Shaken	19	+4
	119	0·50	6·1	—	Shaken	21	+2
	146	0·56	6·5	Aged	Static	15	+5

* Prior to inoculation with slope organisms, the medium had a pH of 6·5.

The effect observed when cultures are shaken during the ageing process is not a result of increased aeration, because organisms from a culture aged under N_2 on a shaker are no more sensitive to the peptide than are organisms from an aerobic culture aged whilst shaking. Rather, the effect of shaking during the ageing process appears to be related to its effect on the pH of the culture. Indeed, the development of the marked sensitivity to the peptide requires prolonged exposure of the organisms to an acid medium, about pH 5·5 (Table 2). This also explains why organisms aged in static cultures of the well-buffered medium C (Table 1) behave like young organisms rather than like organisms aged in static cultures of the poorly buffered medium A. It should be noted that medium C *per se* does not prevent inhibition of growth by the peptide when the inoculum consists of slope organisms.

Organisms taken from complete-broth cultures resemble young organisms, rather than slope organisms, indicating that the types of bacteria produced during a 24 hr. incubation period in broth and on the 'broth+agar' mixture may differ as widely as do young and aged organisms from stationary cultures in the much simpler synthetic medium A. As will become evident from the subsequent discussion, this

similarity between slope and aged organisms is seen only in the growth tests, for these two types of cells differed significantly in other tests.

Hydrolysis experiments. To determine the relative peptide-splitting ability of bacteria grown under different conditions, a study was made using freshly harvested whole organisms and cell-free extracts prepared from them.

Table 3. *Hydrolysis of glycyl-*L*-leucine by whole-cell preparations of the leucine auxotroph of* Escherichia coli K-12

Complete reaction mixtures contained 2 mM-peptide and the specified amount of bacterial nitrogen (determined by micro-Kjeldahl analysis) in the form of freshly harvested organisms. Data used to estimate '% hydrolysis observed' were obtained by the photometric ninhydrin procedure. The values are, at best, minimal ones because no corrections were made for the loss of glycine that occurs, via deamination, during the longer incubation periods.

Preparation of organisms		Bacterial N (μg./ml. reaction mixture)	% Hydrolysis observed				
			Mixtures incubated in static flasks for:				
Growth conditions	Cell type produced		2 hr.	6 hr.	15½ hr.	18 hr.	24 hr.
Static	Young	32	14	36		70	63
Shaken	Young	29	13	38	53	60	65
Static	Aged*	37	0	0		12	10
Shaken	Aged*	32	0	10	20	26	38
			Mixtures incubated in shaken flasks for:				
			2 hr.	6 hr.	12½ hr.	18½ hr.	24 hr.
Shaken	Young	20	26	42	50	52	50
	Slope	21	4	15	22	22	12

* 'Aged cells' listed on lines 3 and 4 came from the cultures used to provide samples of 'young cell' listed on lines 1 and 2, respectively.

Whole-cell preparations of young organisms were found to hydrolyse glycyl-L-leucine rapidly; and young organisms taken from static and shaken cultures have the same activity (Table 3). However, shaking of the hydrolysis reaction mixtures causes an approximate doubling of the initial (first 2 hr.) rate of hydrolysis over that observed in static mixtures. Additional experiments with young organisms (in which samples of the reaction mixtures were removed for analysis at hourly intervals) indicated that this rapid rate is maintained for about 4 hr. in shaken mixtures; under static conditions, the initial (slower) rate is maintained for at least 6 hr.

The fact that, even after 24 hr., the observed extent of hydrolysis does not reach 100% is due to the deamination of the free glycine that accumulates during the hydrolysis. Such deamination occurs very slowly, e.g. in the presence of 2 mM-glycine, the amino acid is deaminated by whole organisms providing 20–40 μg. of bacterial nitrogen/ml. of reaction mixture at an initial rate of *c.* 2%/hr. (unpublished, S. Simmonds and J. Pascale). Valid comparisons of the hydrolytic action of various cell types may be based on the data obtained by the photometric ninhydrin procedure for the initial 6 hr. incubation period. This was established in experiments similar to those in Table 3 but with glycyl-[^{14}C]L-leucine as the substrate. Samples of the reaction mixtures were taken at intervals and analysed by the ninhydrin method and by paper chromatography followed by ^{14}C-measurements. In an experi-

ment with young organisms, the percentage hydrolysis at 2, 6, 11, 19 and 24 hr. was 25, 48, 74, 90, and 89%, respectively, by ^{14}C-analysis and 20, 46, 74, 80 and 76% respectively, by ninhydrin analysis. With aged organisms, samples at 2, 6, 11, 21 and 24 hr. showed 2, 2, 4, 19 and 17% hydrolysis by the ^{14}C-method, and 0, 0, 0, 5 and 6% hydrolysis by the ninhydrin method.

Whole-cell preparations of slope organisms and of organisms aged in static cultures of medium A lack the high enzymic activity of young organisms, but organisms aged in shaken cultures show more peptidase activity than those aged in static cultures (Table 3).

Table 4. *Hydrolytic activity of whole-cell preparations and cell-free extracts of the leucine auxotroph of* Escherichia coli K-12

'Young cells' were from a shaken culture and 'aged cells' from a static culture. Each washed whole-cell preparation was divided into two equal portions, one of which was tested directly and the other used to prepare the cell-free extract. Reaction mixtures containing aged-cell preparations were incubated statically; the other mixtures were shaken. Other procedures were as noted in Table 3.

Cell type and preparation used	Bacterial N (μg./ml. reaction mixture)	% Hydrolysis observed after: 2 hr.	6 hr.	11 hr.	14 hr.	18 hr.	21 hr.	23 hr.
Aged cells	13	4	6	—	16	6	5	—
Aged extract	7	14	42	—	—	68	65	—
Aged cells	91	2	22	—	74	47	—	52
Aged extract	40	48	87	—	98	94	—	100
Young, extract	35	48	100	—	100	78*	—	26*
Slope cells	12	14	20	30	—	—	—	—
Slope extract	4	10	16	24	—	—	—	—
Slope cells	81	27	99	100	—	—	—	—
Slope extract	18	33	71	96	—	—	—	—

* Values indicate the disappearance of 0·44 μmole of free amino acid/ml. reaction mixture during the period from 14 to 18 hr., and 1·48 μmole from 14 to 23 hr. (based on the presence of 2 μmole of free glycine + 2 μmole of free leucine/ml. at 14 hr.).

When cell-free extracts were tested (Table 4), it became apparent that extracts of organisms aged in static cultures contain as much hydrolytic activity as extracts of young organisms. However, the preparation of extracts from slope organisms does not produce the marked increase in the amount of enzymic activity observed with aged organisms.

A further comparison of young and aged organisms was made using lyophilized preparations in an attempt both to circumvent any permeability barriers present in freshly harvested bacteria and to reduce the loss of free glycine by deamination (see Simmonds & Griffith, 1962). Again, the young organisms showed significantly more hydrolytic activity than the aged organisms (Table 5). However, the activity of the lyophilized preparation of aged organisms appears to be greater than that of the fresh preparation (cf. Tables 3 and 5).

Uptake experiments. Because the results of the hydrolysis experiments might be taken as evidence that whole organisms from cultures aged statically are relatively

Table 5. *Hydrolytic activity of lyophilized preparations of the leucine auxotroph of* Escherichia coli K-12

Complete reaction mixtures contained, per ml., 0·3 mg. of lyophilized organisms (from static cultures) providing 36 μg. and 42 μg. of bacterial-nitrogen as young and aged organisms, respectively. Reaction mixtures were incubated statically; other procedures as noted in Table 3. The 'corrected' % hydrolysis was calculated from the 'observed' value plus the amount of NH_3 formed, based on the assumption that the sum of leucine + glycine + NH_3 (in μmole) is equal to the amount of glycylleucine (in μmole) hydrolysed.

		Analyses after incubation for:				
Cell type	Measurement	2 hr.	6 hr.	12 hr.	18 hr.	24 hr.
Young	NH_3 (μmole/ml.)	0	0	0·06	0·30	0·70
	% Hydrolysis					
	observed	14	42	60	55	61
	calculated	14	42	63	70	96
Aged	NH_3 (μmole/ml.)	0	0	0·02	0·24	—
	% Hydrolysis					
	observed	0	27	41	41	—
	calculated	0	27	42	53	—

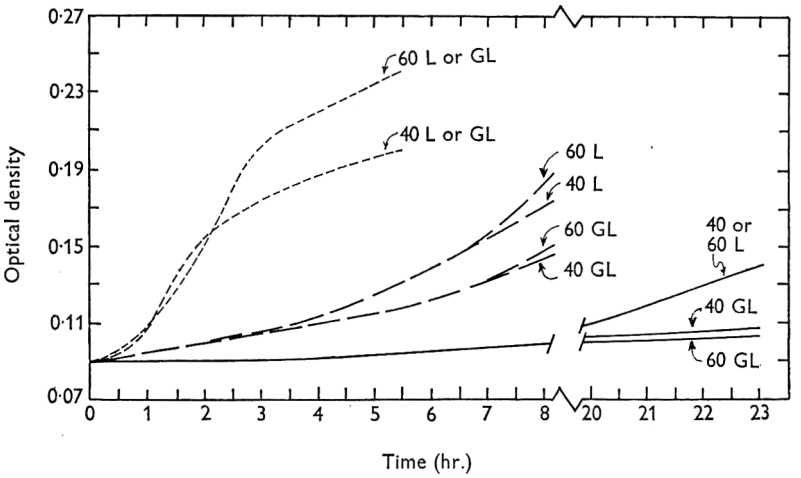

Fig. 1. Growth curves for cultures of the leucine auxotroph of *Escherichia coli* K-12 tested under conditions similar to those used in uptake experiments. Young organisms (dotted lines), slope organisms (dashed lines) or aged organisms (solid lines) were suspended in medium A (containing threonine), and the suspensions were supplemented with 40 μM- and 60 μM-L-leucine (curves 40 L and 60 L) or with 40 μM- and 60 μM-glycyl-L-leucine (curves 40 GL and 60 GL). Growth of young organisms is not delayed in the presence of 100 or 200 μM-peptide; with slope and aged organisms, the bacteriostatic effect increases as the peptide concentration is raised, and, at the 200 μM level, there is no optical density (absorbancy) increase for the aged-organism culture in 35 hr.

impermeable to glycylleucine, the ability of these bacteria to take up the peptide was tested directly, and was compared with that of both young and slope organisms.

Before the uptake of labelled compounds could be studied, it was necessary to determine the 'sensitivity' of the bacteria to glycylleucine under conditions where the medium initially contains the very large numbers of bacteria required for the uptake experiments. Peptid concentrations of 40–200 μM were found to have no

inhibitory effect on the growth of young organisms, but concentrations of 40–60 μM are bacteriostatic both to slope organisms and to aged organisms (Fig. 1). The initiation of growth on free leucine requires prolonged incubation when the medium contains slope organisms or organisms aged in static cultures of medium A; young organisms begin to grow after only a short lag period (15–30 min. after the addition of leucine to an otherwise complete medium).

For the uptake experiments, the labelled leucine and glycylleucine were used at initial concentrations of 50 or 100 μM in medium A devoid of threonine. Such an uptake medium presumably is incapable of supporting growth and protein synthesis because it lacks threonine. Preliminary tests indicated that it does not support growth (determined by optical density measurements), but a significant portion of the isotopic carbon taken up by the organisms may be present in the cell fraction insoluble in cold 13% (w/v) trichloroacetic acid. Such 'protein synthesis' can be inhibited by the addition of chloramphenicol to the uptake medium (Table 6, Expts. 1 and 2). Apparently, the lack of an exogenous source of threonine, which is an essential growth factor for these bacteria, does not prevent completely the incorporation of leucine into protein when that process is not inhibited by chloramphenicol (cf. Levine & Simmonds, 1960).

Table 6. *Uptake of labelled compounds by the leucine auxotroph of* Escherichia coli K-12

Expt. no.	Cell type used	Time of sampling	Isotope content (mμmoles of compound/cell-sample)			
			Leucine in medium*		Glycylleucine in medium†	
				+ Chloramphenicol		+ Chloramphenicol
1	Aged	13 hr.	4·8‡	1·4	—	—
2	Young	15 min.	1·8	0·55	3·3	1·2
		30 min.	4·0	0·16	4·9	1·3
3	Young	2 min.	—	0·40	—	0·42
		4 min.	—	0·69	—	0·46
		6 min.	—	0·19	—	0·60

* Uptake media contained 100 μM-[^{14}C]L-leucine of specific activity 14·7 counts/min./mμmole in Expts. 1 and 2, and 15·6 counts/min./mμmole in Expt. 3.

† Uptake media contained 100 μM-glycyl-[^{14}C]L-leucine of specific activity 17·4 and 20·6 counts/min./mμmole in Expts. 2 and 3, respectively.

‡ Cell-fraction insoluble in cold trichloroacetic acid contained ^{14}C equivalent to 3·1 mμmole of leucine.

The 13-hr. incubation period used in Expt. 1 of Table 6 was chosen because it is equivalent to the time required before the optical density of a control culture containing threonine showed a detectable rise. In the initial series of uptake experiments, measurements were made at the time corresponding to the end of the lag period of the growth cycle of each cell type in a medium containing leucine and threonine (see Fig. 1), i.e. when all organisms are equally capable of initiating growth in a complete medium. However, as the lag time for aged organisms was so long, it seemed probable that incubation of non-growing cultures with chloramphenicol for this period might cause extensive damage to the bacteria. Furthermore,

the auxotroph often 'loses' its requirement for threonine on prolonged incubation in the absence of that amino acid. Both these factors would invalidate comparison of the aged organisms with other cell types. Consequently, it was decided to make uptake measurements only during the first 10 or 15 min. after the first exposure of the organisms to the labelled compounds.

It should be noted that, in Expt. 2 of Table 6 where young organisms were incubated in the presence of chloramphenicol, the amount of peptide-leucine taken up at 15 min. and at 30 min. is the same, but the amount of free leucine taken up

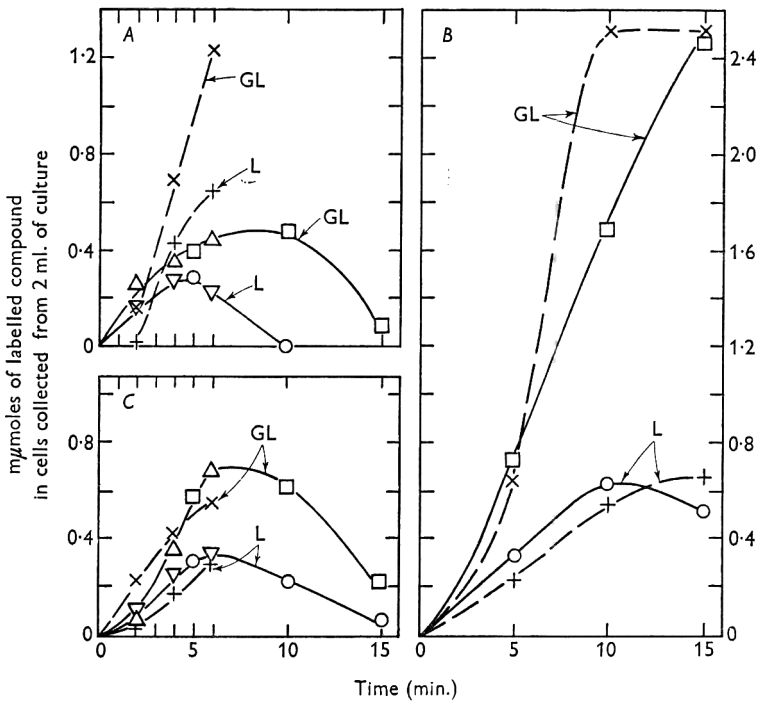

Fig. 2. Uptake of labelled L-leucine (curves L) and glycyl-L-leucine (curves GL) by the leucine auxotroph of *Escherichia coli* K-12. Young organisms (*A*), aged organisms (*B*), or slope organisms (*C*) were suspended in basal medium A devoid of threonine in the absence (dashed lines) and presence (solid lines) of 100 μg. chloramphenicol/ml. The suspensions then were supplemented with 50 μM-labelled compound. In *A* and *C*, data for the three sets of points: × and +; △ and ▽; ○ and □; were obtained in three separate experiments. All data in *B* are from a single experiment. The specific activity of the [^{14}C]leucine varied from 35·9 to 41·0 counts/min./mμmole, and that of the glycyl-[^{14}C]leucine from 32·2 to 36·9 counts/min./mμmole.

is significantly greater at 15 min. than at 30 min. Such a decrease in the isotope content of young organisms given free leucine has been observed repeatedly, and starts soon after the uptake of the amino acid has reached a maximum (Table 6, Expt. 3 and Fig. 2*A*).

Experiments using 50 μM-test compounds (Fig. 2*A*), like those summarized in Table 6 for the 100 μM level, show that young organisms take up glycylleucine at a rate equal to, or slightly faster than, the rate at which they take up free leucine. In uptake media containing chloramphenicol, the initial rise in isotope content is

followed by an immediate decline when the test compound is the free amino acid, and by a 'plateau period' preceding the decline when the test compound is the dipeptide.

In general, the uptake of leucine and glycylleucine by slope organisms (Fig. 2*C*) resembles that by young organisms. However, the data indicate two major differences: first, the slope organisms apparently do not incorporate significant amounts of ^{14}C into protein during a short exposure to labelled test compounds in the absence of chloramphenicol. Secondly, compared to young organisms, slope organisms appear to take up somewhat more glycylleucine and to lose the ^{14}C derived from the peptide at a somewhat slower rate. Slope organisms also appear to lose ^{14}C derived from exogenous leucine at a slower rate than do young organisms.

Aged organisms differ significantly from the other types (Fig. 2*B*). Unlike young organisms, they do not appear to incorporate leucine into protein in the absence of chloramphenicol in 15 min. After very long exposure (13–15 hr.) to labelled leucine (or glycylleucine) the ^{14}C content of organisms incubated in the absence of chloramphenicol (equivalent to 3–17 mμmole leucine) may be 3–20 times higher than that of organisms incubated in the presence of the antibiotic (see also Table 1, Expt. 1). Unlike both young and slope organisms, aged organisms continue to take up leucine for at least 10 min. after the initial exposure to the amino acid. This prolonged uptake of free leucine was observed also in a preliminary experiment in which the 5 and 10 min. values for organisms incubated in the presence of chloramphenicol corresponded to 0·24 and 0·35 mμmole of leucine, respectively.

The uptake of glycylleucine-carbon by aged organisms is much greater than that by young or slope organisms. Moreover, there appears to be no loss of isotope from the aged bacterial cells during the uptake period, as judged by the data in Fig. 2*B*, and also by data from the preliminary experiment where the 5, 10, and 15 min. values corresponded to 0·41, 0·83 and 1·58 mμmole of labelled compound respectively.

DISCUSSION

Whole-cell preparations of the leucine auxotroph of *Escherichia coli* strain K-12 that consist of organisms grown on complete-agar slopes for 24 hr. or aged for several days in static cultures of an acidified synthetic medium lack the high hydrolytic activity toward glycyl-L-leucine shown by young organisms. The low peptidase activity is paralleled by a marked sensitivity to the bacteriostatic effect of the peptide on the initiation of growth when the growth medium contains glycylleucine in place of leucine. As might be expected from their relatively better growth response, organisms aged in shaken cultures (whose pH does not fall below *c.* pH 6) show a greater peptidase activity than organisms aged in static cultures. Thus, ageing in the static liquid cultures would appear to be accompanied by the same changes in the metabolic capabilities of whole bacteria that characterize growth on the slopes, but the changes seem to take place at a faster rate when the organisms are closely packed on the surface of a slope.

The similarity between slope and aged organisms no longer is evident when peptidase activity is measured using cell-free extracts. The amount of activity extracted from slope organisms is approximately equal to that of the whole bacteria, whilst the activity in the extracts of aged organisms is markedly greater than that

demonstrable in whole-cell preparations. Although the inability of slope organisms to grow readily on glycylleucine may result from their relatively low peptidase activity, a similar explanation cannot be offered for the poor growth of aged organisms.

Failure to detect high peptidase activity in whole-cell preparations of aged organisms might be attributed to one, or both, of two factors: either the whole bacteria may be relatively impermeable to the dipeptide (i.e. lack the ability to take up glycylleucine from the medium) or the bacterial cells may contain a constituent which inhibits the action of the hydrolytic enzyme *in vivo* but whose inhibitory effect is lost during the preparation and testing of the extracts. The data obtained in the uptake experiments indicate that aged organisms do not have a 'permeability barrier' which prevents the exogenous peptide from reaching the interior of the cells and which is eliminated when the cell extracts are made. Indeed, the existence of such a permeability barrier is unlikely because glycylleucine has a bacteriostatic effect on the leucine auxotroph cultured in the presence of free leucine, and on other *Escherichia coli* strains that do not require an exogenous source of either leucine or glycine for growth. Presumably, the peptide must be taken up by these bacteria in order for it to inhibit their growth. The inability of whole-cell preparations of aged organisms to catalyse the hydrolysis of the peptide seems, therefore, to be related to the presence of a cell constituent that is produced during the ageing process and that exerts an inhibitory effect upon the peptidase within the intact bacterial cells. Further evidence in support of this hypothesis has been obtained in preliminary experiments in which 'heated extracts' of aged organisms were prepared by centrifugation of a suspension of whole bacteria in 0·9% NaCl solution that had been heated at 100° for 10 min. Addition of this extract to reaction mixtures containing either whole young organisms or the usual cell-free extracts of aged organisms caused a significant decrease in the initial rate of glycylleucine hydrolysis.

All three types of organisms readily accumulate leucine-carbon during the 5–10 min. following the initial exposure of the bacteria to labelled leucine or glycylleucine. The differences observed in the uptake experiments with the three cell types may be related directly to their respective metabolic states at the time of testing. Thus, young organisms are capable of initiating growth almost immediately upon resuspension in a complete growth medium (i.e. a medium containing threonine and leucine). In a threonine-deficient medium containing chloramphenicol, such organisms accumulate ^{14}C from leucine for only a short time, after which the ^{14}C-content of the organisms falls rapidly. One possible explanation for the drop is suggested by the finding of Cohen & Rickenberg (1956) that the [^{14}C]valine concentrated by non-growing organisms of *Escherichia coli* is displaced by isoleucine and leucine. In agreement with this observation, we have found the amount of labelled carbon from exogenous leucine (or glycylleucine) which is taken up by young cells to be reduced about 50% when the uptake medium also contains L-isoleucine. Consequently, there may occur a 'displacement' of the labelled leucine taken into the bacterial cells by the unlabelled isoleucine and valine that is formed within the cells from the glucose and N-sources in the uptake medium.

It is reasonable to assume that the intracellular cleavage of the dipeptide taken up by the young organisms yields free leucine and this subsequently is lost from the

bacterial cells like the cellular leucine derived directly from the free leucine of the medium. An analogous rapid intracellular hydrolysis of dipeptides has been found with other strains of *Escherichia coli* (Levine & Simmonds, 1960, 1962) and with *Lactobacillus casei* (Leach & Snell, 1960); some of these bacteria take up glycine-containing peptides at a faster rate than they take up the constituent amino acids. With *L. casei*, also, resting organisms incorporate amino acid-carbon (derived from exogenous amino acids or dipeptides) into protein, and the data presented by Leach & Snell (1960) indicate that the free amino acid in the trichloroacetic acid-soluble portion of the cells (like the isotopic-carbon in our chloramphenicol-treated organisms) first increases and later decreases in amount. A similar rapid increase followed by a drop in the size of an amino acid 'pool' was reported for the organisms in growing cultures of *E. coli* (Bolton *et al.* 1957), but has not, to our knowledge, been found in non-growing cultures (Britten, Roberts & French, 1955; Cohen & Rickenberg, 1956; Levine & Simmonds, 1960).

Unlike young organisms, slope organisms do not appear to incorporate leucine-carbon into protein in the absence of chloramphenicol. This may be explained by the fact that slope organisms grow much more slowly than young organisms in the complete medium, and so may be assumed to be less capable of the synthetic reactions associated with growth. Their lower synthetic capacities also would permit only a limited formation of isoleucine and valine, which would explain why slope organisms also appear to lose ^{14}C derived from leucine in the presence of chloramphenicol at a somewhat slower rate than that seen for young organisms. The ability of slope organisms to take up more peptide-carbon and to retain it better than do young organisms probably reflects their lower peptidase activity if, as suggested above, the decline in the ^{14}C-content of the organisms results from the loss of leucine liberated by the intracellular hydrolysis of glycylleucine.

The carbon of free leucine is retained better by aged organisms than by the other cell types studied, although the aged organisms do not incorporate it into protein in the absence of chloramphenicol. Because aged organisms can initiate growth in a complete medium only after a very extended lag period, they may be assumed to carry out synthetic reactions at a very slow rate. This would decrease the displacement of the free leucine by isoleucine and valine formed within the bacterial cells, and also would limit the synthesis of protein. The extensive and prolonged uptake of peptide-carbon observed with aged organisms provides further support for the view that the loss of peptide-carbon from the bacterial cells occurs only after the glycylleucine has been hydrolysed. Thus, because whole-cell preparations of aged organisms do not hydrolyse glycylleucine, the bacteria continue to accumulate ^{14}C, and build up intracellular concentrations of isotope far exceeding those attained in the presence of exogenous leucine. From the comparison of the hydrolytic activity and uptake response of young and aged organisms, it is clear that the failure of the latter to grow readily on glycylleucine results from the inhibition of the activity of the intracellular peptidase by some other cell constituent. On the other hand, failure of slope organisms to grow readily on the peptide appears to be a consequence of their relatively low peptidase activity. Together, these data emphasize the fact that the extent to which glycylleucine may inhibit bacterial growth depends directly upon the metabolic state and internal environment of the organisms tested.

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On the Nature of Competence of Transformable Streptococci

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SUMMARY

Transformable streptococci produce an exocellular factor provoking competence under certain conditions. Non-competent cultures become competent upon addition of this factor. The kinetics of conversion is concomitant with an enzymic reaction; the process is time and temperature dependent and the factor itself is heat sensitive. The action of the hypothetical enzyme on cells of a non-transformable streptococcus results in provocation of competence.

INTRODUCTION

The term competence is defined here as the ability to undergo transformation. Some workers define this state in terms of capacity of cells to take up DNA irreversibly and to undergo subsequently transformation. There is, indeed, no real difference between the definitions since DNA uptake is a necessary condition of genetic transformation. The definition of biochemical conditions favouring competence is of considerable interest. Transformability of bacteria is limited to some strains of a small number of species. Without an understanding of the nature of competence, extension of the phenomen of transformation to more bacterial species can only be a matter of chance. The physiological state of competence seems to be in some way connected with the mechanism of DNA penetration through the cell wall. Two hypotheses have been proposed to account for penetration: one termed the 'localized protoplast' hypothesis and the other defined as the 'enzymic receptor' hypothesis (Ravin, 1961). The 'localized protoplast' hypothesis, based mainly on evidence provided by Thomas (1955), regards the competent cell as one having during some period of its life cycle naked areas on the surface through which DNA can penetrate. According to the second hypothesis, competent cells produce on their surface an enzyme which catalyses DNA uptake (Fox & Hotchkiss, 1957). Pakula, Piechowska, Bankowska & Walczak (1962) reported that sterile supernatant fluids from cultures of competent streptococci contain a factor which provokes competence of homologous and related cells. Further work, presented in this paper, has shown that upon addition of this factor, transformation can occur in a medium containing neither serum nor albumin, i.e. in conditions in which transformation of streptococci never occurs or is extremely infrequent. Moreover, the factor produced by the strain Challis, a group H Streptococcus, provokes transformation of the non-transformable strain Wicky of the same serological group. The conversion of non-competent cells into competent ones seems to be concomitant with an enzymic reaction.

METHODS

Organisms. The following streptomycin-sensitive strains of group H Streptococcus were used as recipients: Challis, 3437/48, Wicky. The action of the provoking factor was also tested on some other strains of group H Streptococcus as well as on strains of *Streptococcus sanguis*, type I, type II and type I/II. All the group H streptococci were obtained from the Streptococcal Reference Laboratory, Colindale, London. The strains of *S. sanguis* were given us by Dr R. Wahl of the Pasteur Institute in Paris.

The strains Challis and *Streptococcus sanguis*, type I/II, can be transformed to streptomycin-resistance with rather high efficiencies and have been used in this laboratory in transformation studies (Pakula, Hulanicka & Walczak, 1958). Strain 3437/48 was recently found to be transformable.

Media. Two media were used for the production of transformants and of the factor which provokes transformation. One medium (ST) contained beef heart extract, prepared as for Todd–Hewitt broth (1932), Difco neopeptone 1 %, charcoal-adsorbed Difco yeast extract 0·5 %, glucose 0·2 %. The second medium (ET 3) contained distilled water, Difco neopeptone 1 %, charcoal-adsorbed Difco yeast extract 0·5 %, sodium chloride 0·85 %, glucose 0·2 %. Both media were at pH 7·4–7·5.

Transforming DNA. One DNA preparation, derived from a mutant of strain Challis resistant to 2 mg. streptomycin/ml., was used throughout this work. The efficiencies of the interspecific transformations to streptomycin-resistance of the heterologous streptococcal strains with this DNA are of the same order of magnitude as those obtained with the corresponding homologous DNA preparations. The procedure of DNA extraction and purification was reported elsewhere (Pakula *et al.* 1962).

Competent cells. Under normal conditions, i.e. without addition of the provoking factor, competent cells of the three transformable strains: Challis, 3437/48 and *Streptococcus sanguis*, type I/II, cannot be produced in either of the above two media, unless swine serum, previously heated for 30 min. at 62° to destroy DNAase activity, or albumin is added, this is absolutely necessary to produce any significant transformation. However, upon addition of active culture filtrates or of crude concentrates thereof, transformation can be obtained, in some instances, in medium ET 3 containing neither serum nor albumin. In order to obtain non-competent cells for the provocation test, 50 ml. of this medium was inoculated with 2·5 ml. of an 18 hr. blood broth culture previously diluted 1/250. With this size of inoculum initial concentrations of 5 to 7×10^4 cocci/ml. were found. After 3 hr. of growth at 37° in aerobic conditions the non-competent culture was converted to competence by addition of the provoking factor as described below.

Preparation of active supernatant fluids and of crude concentrates of the provoking factor. Bacteria grown in any medium which permits transformation of a given strain produce the factor which enhances or provokes competence. The presence of this factor seems to be a minimum requirement for competence of a streptococcal culture. Cultures were centrifuged and the supernatant fluids were filtered through a G 5 glass filter and tested for sterility.

Crude concentrates of factor were obtained by precipitation with ammonium sulphate or ethanol. A cellophan tube was filled with a given volume of the sterile supernatant fluid and placed in a glass vessel containing 10 volumes of a saturated solution of ammonium sulphate. After 18–24 hr. at 4° a precipitate was formed within the tube. Ethanol precipitates were formed after addition of 1 volume of supernatant fluid to 8 volumes of cold ethanol (−10°). Both kinds of precipitates were dissolved in 0·01 M-phosphate buffer (pH 7).

Activity tests of provoking factor. Induction of competence was demonstrated under two different conditions: (1) in medium ET3 not containing either serum or albumin; (2) in medium ST − serum. In the first case, competence appeared only on adding active supernatant fluid, or concentrate. As concerns the second case, it was shown by Pakula *et al.* (1962) that the time of appearance of competence of streptococci, in media containing serum or albumin, was dependent on the inoculum size. The smaller the inoculum the later the appearance of competence. The activity of the provoking factor can, therefore, be shown in cultures started with a small initial inoculum when the factor is added before natural competence occurs.

Activity of the provoking factor was measured by exposing to it for 30 min. a 3 hr. non-competent culture of an initial density mentioned above, 5 μg. DNA/ml. was then added and after 10 min. any non-incorporated DNA was destroyed by adding DNAase. Bacteria were grown for the next 2 hr. to allow phenotypic expression of the acquired streptomycin-resistance character. Transformants were scored on blood agar plates containing 250 μg. streptomycin/ml.

RESULTS

Development of competence and production of the provoking factor

Figure 1 illustrates the time-dependence of competence and the production of provoking factor by a culture of strain Challis in ST medium + 5% swine serum. With an initial concentration of 0·5 to 1×10^6 cocci/ml., maximum competence was achieved after about 3 hr. The frequency of transformants at this time was 8–22% of the total number of cocci. After incubation for 7–8 hr. competence disappeared completely.

At the times indicated in Fig. 1, samples of culture were collected, sterile supernatant fluids prepared from them and their ability to provoke competence tested on bacteria cultivated in medium ET3 without serum or albumin. As mentioned above, under these conditions no transformants are produced in absence of provoking factor. It can be seen in Fig. 1 that the activity of culture supernatant fluids increased initially with the increase of competence. However, while competence disappeared at the end of the logarithmic phase of growth, the activity of culture supernatant fluids prepared after incubation for 17 hr. did not differ markedly from those prepared at the time of maximum competency.

The addition of 5–10% (v/v) of an active supernatant fluid to bacteria incubated in medium ET3 for 3 hr. followed by incubation of the mixture for 30 min., resulted in transformation of 5–15% of the entire population.

Medium ST and medium ET3, supplemented with serum or albumin, are both suitable for transformation of organisms of strain Challis and for the production of provoking factor. Transformation of *Streptococcus sanguis* type I/II was also

observed in these media but only in the presence of serum, not of albumin. Medium ET3, supplemented with serum, permitted transformation of strain 3437/48. The efficiencies of transformation of *S. sanguis* type I/II and of strain 3437/48, as a percentage of entire population, were 3–8 % and 2–4 %, respectively.

The activity of supernatant fluids and of crude concentrates of provoking factor

Figure 2 illustrates the provocation of competence of organisms of strain Challis, grown in medium ET3 without serum or albumin, when homologous supernatant fluid and crude concentrates were added. The concentrates were obtained by precipitation with ammonium sulphate, as mentioned above, or with 8 volumes of ethanol, an amount found to be optimal. It may be seen that the effect was dependent on the amount of provoking factor added. Curves C and E, illustrating

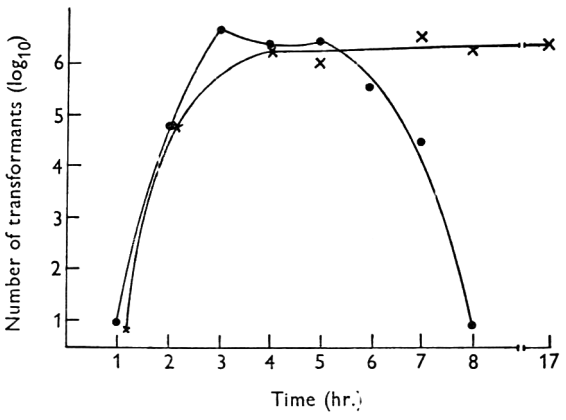

Fig. 1

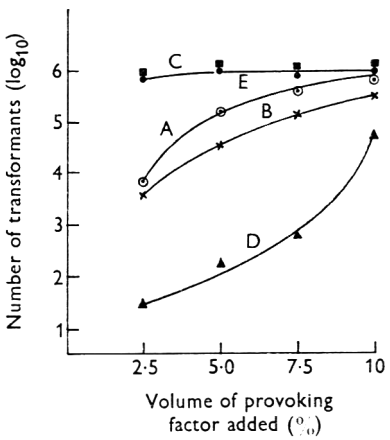

Fig. 2

Fig. 1. Development of competence of Streptococcus strain Challis, in medium ST+ serum, and competence-provoking activities of supernatant fluids prepared at times indicated. The competence-provoking activity of supernatant fluids was tested on homologous organisms grown in medium ET3. ●—●, competence; ×—×, competence-provoking activity of the supernatant fluid.

Fig. 2. Competence-provoking activities of a supernatant fluid and of crude concentrates of provoking factor. A, supernatant fluid; C, ammonium sulphate precipitate dissolved in 1/10 of the initial volume; E, ethanol precipitate dissolved in 1/10 of the initial volume; B, ammonium sulphate precipitate dissolved and diluted to the initial volume of the supernatant fluid used for precipitation; D, ethanol precipitate dissolved and diluted to the initial volume of the supernatant fluid used for precipitation.

the development of competence following the addition of the concentrates, follow the same line. The smallest amounts of the concentrates used were found to be saturating. From these curves it might be concluded that the efficiencies of both methods of concentration are equal. However, this is not true, as can be seen from curves B and D, which show the activities of concentrates diluted to the initial volume of the supernatant fluid used for concentration. It is evident that precipitation with ammonium sulphate was much more effective than precipitation with ethanol.

Heat sensitivity of the provoking factor

Concentrates of the factor which provokes competence were diluted either in medium ET3 or in 0·01 M-phosphate buffer (pH 7) or in saline. Samples of all these solutions were heated for 10 min. at various temperatures (see Fig. 3) and their ability to provoke competence tested on strain Challis organisms grown in medium ET3 without serum or albumin. Competence in this condition was solely due to addition of provoking factor since transformation was never observed in controls not supplemented with this factor. Obviously, non-saturating amounts of provoking factor were added in order to observe any decline of activity caused by heating. Samples of provoking factor diluted before heating in medium ET3 were more resistant than those diluted in buffer or saline. The provoking factor seems to be protected by some organic compounds present in the medium.

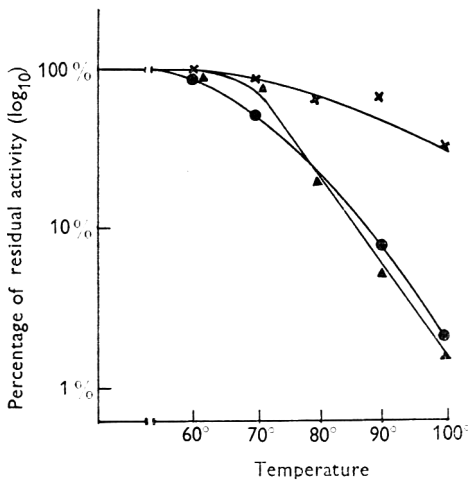

Fig. 3

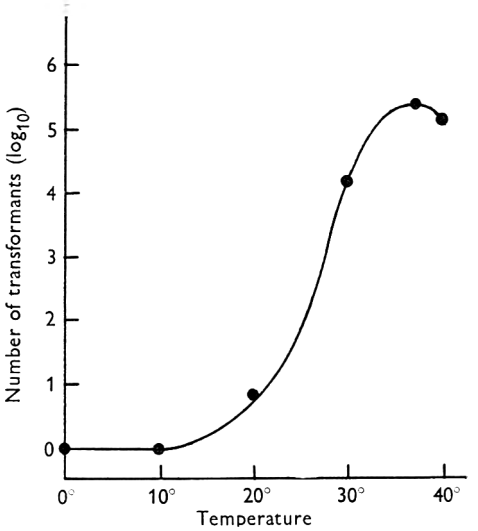

Fig. 4

Fig. 3. Heat sensitivity of competence-provoking factor. The activity of the unheated diluted concentrate is indicated as 100 %. ×—×, Concentrate diluted in medium ET3 before heating; ●—●, concentrate diluted in buffer before heating; ▲—▲, concentrate diluted in saline before heating. All samples were heated 10 min. at the temperatures shown.

Fig. 4. Dependence of activity of the competence-provoking factor on temperature.

Temperature dependence of the activity of the provoking factor

A 3 hr. culture of strain Challis growing in medium ET3 was divided into several samples, each adjusted to one of the temperatures indicated in Fig. 4 and supplemented with the factor which provokes competence. This factor was allowed to act on organisms at the indicated temperatures for 30 min. At the end of this period, all the samples were transferred to a 37° water bath, exposed to DNA for 10 min. and incubated for 2 hr. to permit phenotypic expression of transformants. As shown in Fig. 4, the provoking factor was not active at 0° and 10°; maximum activity was at 37°. Temperatures higher than 40° could not be used because they affected growth and viability.

Time of action of provoking factor and efficiency of transformation

A 3 hr. culture of strain Challis growing in medium ET3 was divided into equal samples. Equal amounts of provoking factor were then added to each sample at various times, so that the bacteria in each sample had been grown for the same time but the cocci were exposed to the provoking factor for different times. After application of the normal procedure, the number of transformants in each sample was determined. As indicated in Fig. 5, the optimal period of action of provoking factor was 25–35 min. The mean generation time of strain Challis organisms in medium ET3 is 33 min.

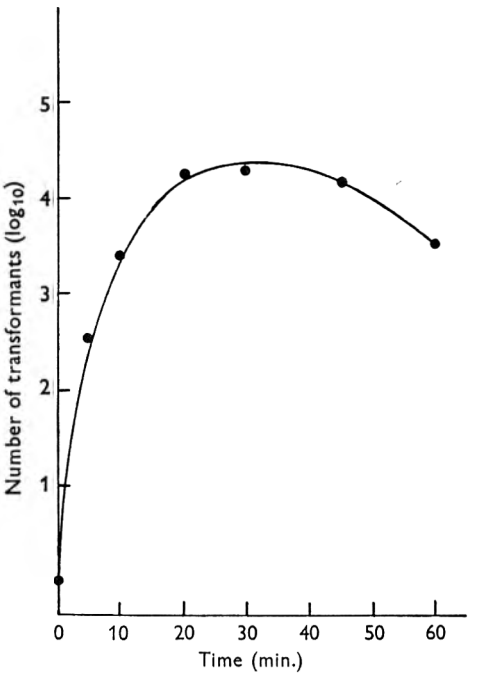

Fig. 5

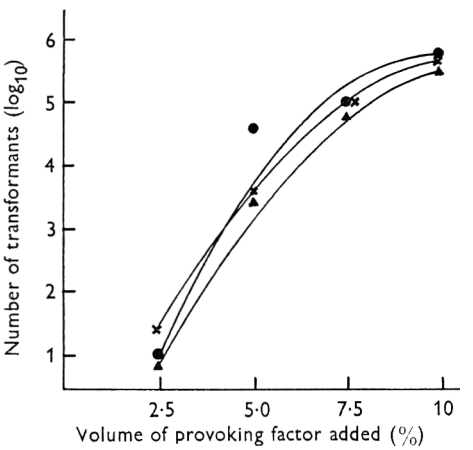

Fig. 6

Fig. 5. Dependence of activity of the competence-provoking factor on the time of contact with the organisms before DNA addition.

Fig. 6. Effect of dialysis on the activity of competence-provoking factor. ×—×, activity of the dialysed concentrate diluted in buffer; ▲—▲, activity of the dialysed concentrate diluted in buffer used for dialysis; ●—●, activity of the non-dialysed concentrate diluted in buffer.

Dialysis of the factor which provokes competence

Visking dialysis tubing, declared to hold back particles of molecular weight greater than 10^4, were filled with a given volume of a concentrate of provoking factor and placed in a glass vessel containing 20 volumes of 0·01 M-phosphate buffer (pH 7). After dialysis at 4° for 48 hr., samples of the dialysis residue were appropiately diluted in fresh buffer and in the buffer which had been used for the dialysis. A non-dialysed sample of the batch of provoking factor concentrate was diluted in the same way. The activity of these samples was tested on organisms

grown in medium ET3. The results, given in Fig. 6, show a slight loss of activity after dialysis. This loss was not restored by dilution of the dialysis residue in the buffer used for dialysis which suggests that no co-factor was necessary for the action of the hypothetical enzyme. This kind of experiment was repeated several times. The loss of activity varied from experiment to experiment but was never very significant. Some variation might have been due to the inaccuracy of the test used for measuring of activity of provoking factor.

The specificity of action of provoking factor

Samples of competence-provoking factor were prepared from cultures of the three transformable strains, Challis, *Streptococcus sanguis* type I/II and 3437/48. Concentrates of these preparations were diluted appropriately and their capacities to provoke transformation tested on these transformable strains as well as on the following non-transformable strains of group H Streptococcus and *S. sanguis*: Wicky, E91/46, Channon, Blackborn, H4, *S. sanguis*, types I and II. Provocation of competence was observed in medium ET3 and in medium ST+swine serum. In the latter medium, organisms grown only for 2–2½ hr. were used for test, that is, before competence naturally occurred or, if it did, was of very low degree.

Table 1. *Specificity of action of competence-provoking factors derived from cultures of transformable strains of Streptococcus: Challis;* S. sanguis *type I/II;* 3437/48

Test organisms grown in medium ET3 without serum or albumin.

	Numbers of transformants in 1 ml. of culture			
		Activity of provoking factors derived from cultures of strains		
Provocation of competence tested on Streptococcus strains	Control without provoking factor	Challis	*S. sanguis* type I/II	3437/48
Challis	0	1×10^6	0	0
S. sanguis type I/II	0	2×10^1	17×10^4	0
3437/48	0	21×10^4	0	0
Wicky	0	52×10^4	0	0
E 91/46	0	0	0	0
Channon	0	0	0	0
Blackborn	0	0	0	0
H_4	0	0	0	0
S. sanguis type I	0	0	0	0
S. sanguis type II	0	0	0	0

Provocation of competence in medium ET3 is illustrated in Table 1. It can be seen that the factor derived from a culture of strain Challis provoked competence of homologous organisms and of organisms of strains 3437/48 and Wicky. The latter strain could not be transformed in the normal way under various conditions found successful with other streptococci. The action of the factor from Challis cultures on *Streptococcus sanguis* type I/II was negligible. The factor from cultures of strain 3437/48 was not active at all under these conditions.

Table 2 illustrates the enhancement of competence in medium ST+serum. In this case, the factor derived from a culture of *Streptococcus sanguis* type I/II

enhanced competence not only of homologous organisms but also those of strain Challis, 3437/48 and Wicky. The factor from cultures of strain 3437/48 acted on homologous organisms and on organisms of the group H streptococci Challis and Wicky, but not on *S. sanguis* type I/II. The same batch of provoking factor was used in the experiments recorded in Tables 1 and 2. It may be pointed out that we succeeded in provoking competence of the probably non-transformable strain Wicky, but we were not able to provoke competence of the other strains of group H streptococci and of *S. sanguis* listed in the Tables.

Table 2. *Specificity of action of competence-provoking factors derived from cultures of the transformable strains of Streptococcus: Challis;* S. sanguis *type I/II; and* 3437/48

Test organisms grown in medium ST with serum.

Provocation of competence tested on Streptococcus strains	Number of transformants in 1 ml. of culture			
	Control without provoking factor	Activity of provoking factors derived from cultures of strains		
		Challis	*S. sanguis* type I/II	3437/48
Challis	4×10^2	2×10^5	88×10^3	1×10^5
S. sanguis type I/II	5×10^2	12×10^4	42×10^4	4×10^2
3437/48	8×10^1	8×10^3	24×10^2	26×10^4
Wicky	0	88×10^3	32×10^3	12×10^2
E 91/46	0	0	0	0
Channon	0	0	0	0
Blackborn	0	0	0	0
H_4	0	0	0	0
S sanguis type I	0	0	0	0
S. sanguis type II	0	0	0	0

DISCUSSION

Experiments presented in this paper show in cultures of transformable streptococci the presence of an exocellular factor which seems to be essential for development of competence. This factor was never found in cultures which were not competent during a given period of growth. On adding competence-provoking factor, transformation of the strain Challis and *Streptococcus sanguis* type I/II was produced in medium ET3 in absence of serum or albumin. It seems clear, therefore, that serum or albumin are not essential for transformation of organisms rendered previously competent. In the absence of serum or albumin and provoking factor, no significant transformation was ever observed. It may be concluded that the presence of serum or albumin is essential for production of provoking factor and, subsequently, for development of competence.

We were not able to transform cells of *Streptococcus sanguis* type I/II in the absence of serum: in this case albumin is not a proper substitute for serum. It is probable that an additional substance, beside albumin, present in serum, plays some role in development of competence in cultures of *S. sanguis*.

The factor which provokes competence seems to be an enzyme. The induction of competence is time and temperature dependent and the factor itself is heat sensitive and does not pass through Visking dialysis tubing. If the factor is an

enzyme, we are dealing with a complicated reaction in which the amount of substrate (the cocci) is increasing during its action. In our experience the addition of even large amounts of provoking factor does not result in conversion of more than about 15% of non-competent cocci into competent ones. It therefore seems likely that in a given period of growth, only a part of the population in a culture is able to react with the provoking factor. Synchronization of division in cultures by cooling before the addition of provoking factor, did not change remarkably the degree level of competence.

The specificity of action of provoking factor produced by the three transformable streptococcal strains was tested on closely related bacteria all of which, including *Streptococcus sanguis* type I/II, contain the C polysaccharide common to group H Streptococcus. In medium ST + serum, reciprocal provocation of competence was demonstrated in most instances (Table 2). However, some specificity of action is evident. Provocation by homologous factors results in production of more transformants than provocation by heterologous factor. The extension of such provocation tests to other streptococci and related bacteria, such as pneumococci, is necessary to determine the range of specificity of action of these factors. Preliminary experiments revealed the probable presence of competence-provoking factor in sterile supernatant fluids of competent cultures of the R36A pneumococcus strain. A similar factor is, probably, also present in competent cultures of *Hemophilus influenzae*, as one may conclude from the data of Goodgal & Herriott (1961). The present findings suggest that development of competence in streptococcal cultures requires the action of an enzyme. The question may, therefore, be asked: what is the substrate of this enzyme? This question cannot be answered until the provoking factor has been purified and its action investigated on some bacterial constituents. First of all, one has to look for it in the cell wall.

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J. gen. Microbiol. (1963), **31**, 135–145
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Cell Structure and Quantitative Gram Stain of *Bacillus megaterium*

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SUMMARY

In a strain of *Bacillus megaterium* the Gram reaction was investigated by comparing its effect on intact bacilli with its effects on bacilli treated with lysozyme in several different ways. The lysozyme-treated bacteria varied from bacilli showing only polar separation of the cell wall from the protoplasm to protoplasts free from cell wall. The uptake of the primary dye (crystal violet) by intact bacteria was higher than that of all lysozyme-depolymerized cell forms. Iodine uptake depended on the previous uptake of crystal violet. Therefore it was lower for the lysozyme-depolymerized forms. Cell wall took up crystal violet and iodine in small quantity. The same dye iodine complex was formed in all cell structures (cell wall and protoplasm). Gram differentiation was obtained with 95% (v/v) ethanol in water, and this was found to extract much less of the dye iodine complex from intact bacteria than from the lysozyme-depolymerized forms. The latter lost about 80–90% of their net dye and iodine content, whereas intact bacilli lost only 20–45%. This was due to the cell wall which formed a barrier to the ethanol extraction of the dye iodine complex, for organisms whose cell walls had been depolymerized or even dissolved by lysozyme during or after the iodine step of the Gram reaction lost much more dye and iodine than intact bacilli. Chemical integrity of the cell wall is a prerequisite for Gram positivity.

INTRODUCTION

Much new information on the mechanism of the Gram reaction has been obtained in the last 10 years by Gram staining bacterial suspensions. Several authors have studied the uptake of the primary dye (crystal violet) by Gram-positive and Gram-negative bacteria. Their results were divergent. Barbaro & Kennedy (1954) and Wensinck & Boevé (1957) analysed the complete Gram reaction of bacteria in suspension. Barbaro & Kennedy (1954) considered that the difference in the uptake of the primary dye which was smaller for Gram-negative bacteria, played the most important role in the Gram differentiation. Wensinck & Boevé (1957) found that the dye uptake was the same for Gram-positive and Gram-negative bacteria. In their view differentiation is obtained by the decolorization which extracts a small amount of dye iodine complex from Gram-positive bacteria and large amount from Gram-negative bacteria. Wensinck & Boevé (1957) and Salton (1961) supposed that the cell wall played an important role in the Gram differentiation. Lysozyme, the enzyme which attacks only the cell wall of some bacterial species, transforms Gram-positive bacteria into Gram-negative forms (Welshimer & Robinow, 1949; Petronelli, 1950; Stähelin, 1953; Gerhardt, Vennes & Britt, 1956). This reversal of the

Gram reaction is one of the first signs of the action of lysozyme upon the bacterial structure (Scherrer, 1961). The present paper reports studies of the action of lysozyme on Bacillus M, a strain of *Bacillus megaterium* (Tomcsik 1950, 1960), and its influence on a quantitative Gram reaction, especially the role of the cell wall in the Gram reaction of the whole organism.

METHODS

Bacillus megaterium strain M was grown on nutrient agar for 13 hr. at 30°; suspensions of it were made in deionized water, in saline (0·9% (w/v) NaCl) or Ringer solution. A standard suspension for each of the three types of suspension was obtained by measuring its turbidity (reading 300 of the Klett–Summerson colorimeter, filter 54).

Preparation of the 'lysozyme-depolymerized forms' of Bacillus M: Lysozyme (crystallized lysozyme; Mann Research Laboratories, Inc., New York 6, N.Y.) to final concentration 50 μg./ml. transformed heat-killed (30 min. at 60°) Bacillus M suspensions into protoplasmic rodlike forms, free from cell wall, the '*small bacilli*' of Welshimer & Robinow (1949) and of Tomcsik & Guex-Holzer (1952). Lysozyme 10 μg./ml. of the standard suspension in Ringer (living bacteria) gave after 30 min. incubation forms which showed only a polar separation of cell wall from protoplasm (Pl. 1, fig. 5), when the activity of lyzozyme was stopped by 38% formaldehyde solution (1 volume of 38% formaldehyde to 5 vol. suspension). Protoplasts were obtained by longer treatment with lysozyme (50 μg./ml. standard suspension of living bacteria). They were fixed in the same manner with formaldehyde.

Dry weight determinations. Samples (30–50 ml.) of standard bacterial suspension or of suspensions of the lysozyme-depolymerized forms were centrifuged in a MSE centrifuge at 5° for 1 hr. at 3000 rev./min. (1760 RCF). The dry weight determination was made at 105° for 5 to 6 hr., each determination was made in triplicate.

Nitrogen determination. A micro-Kjeldahl analysis of Bacillus M in triplicate gave a nitrogen content of 9·08% (dry weight %, mean value).

Preparation of bacterial suspensions or of lysozyme-depolymerized forms for the Gram reaction. The suspensions were centrifuged, the absence of bacilli or of forms in the supernatant fluid was checked by microscopic examination. The sediments were resuspended in the equal volumes of deionized water. These suspensions were all at pH of 6·1–6·3. This method of preparation permitted about the same numbers of bacilli and lysozyme-depolymerized forms in the same volume of suspension to be obtained.

Gram reaction. The reaction was performed after the method of Wensinck & Boevé (1957) with some modifications; the counter-stain was omitted. (1) Crystal violet uptake. Kristallviolett E. Merck, Darmstadt, Germany, No. 1408, batch 136 304, dye content 85% was used for all experiments. A standard solution of 1 mg. dye in 1 ml. deionized water was prepared. A reference curve of the dye concentration was constructed with the Klett–Summerson colorimeter with filter 59, by using dilutions of the standard dye solution in Klett tubes (readings 60–600). The dye uptake was determined as follows: 1 ml. of standard dye solution (= 1 mg. dye) was added to different amounts (1 to 9 ml.) of suspension of bacilli or lysozyme-depolymerized forms. The mixture was left for 10 min. at room temperature and

then centrifuged for 20 min. at 3000 rev./min. The dye concentration of the supernatant fluid was determined in the Klett–Summerson colorimeter with filter 59. The difference between the dye content of the supernatant fluid and the initial 1 mg. dye of the 1 ml. dye standard solution gave the dye uptake.

(2) *Washing step.* The coloured deposits were resuspended in 5 ml. deionized water and then centrifuged for 30 min. at 3000 rev./min. The dye content of the supernatant fluid was then measured.

(3) *Iodine uptake.* 1 g. iodine (Iodum resublimatum pro analysi, Merck) and 2 g. potassium iodate (Kalium iodatum pro analysi, Merck) were dissolved in 800 ml. deionized water. 5 ml. of this solution were added to the coloured and washed sediments of bacilli or cell forms. These suspensions were allowed to stand for 10 min. at room temperature and were then centrifuged for 15 min. at 3000 rev./min. The residual iodine in the supernatant fluid was titrated and iodine uptake determined by difference. Iodine titration was done with N/200 or N/500 $Na_2S_2O_3$ with starch indicator. Experiments have shown that 5 ml. of iodine solution is sufficient to provide excess of iodine with all suspensions used.

(4) *Decolorization.* Ethanol (95% (v/v) in water; 5 ml.) was added to the deposits of bacilli or cell forms after the iodine step. The suspension was allowed to stand for 10 min. and was then centrifuged for 20 min. at 3000 rev./min. The resulting deposits were resuspended in 5 ml. ethanol (95% v/v) and centrifuged again for 15 min. at 3000 rev./min. The dye content of the combined supernatant fluids was determined by the usual colorimetric method (see dye uptake) after adding 2 or 3 drops of concentrated $Na_2S_2O_3$ solution to the supernatant fluids (reduction of the iodine). Iodine of the supernatant fluids was determined by its titration with N/500 $Na_2S_2O_3$ on parallel samples. Because of the crystal violet in the supernatant fluids, the result of this titration was indicated only by a change of the colour of the supernatant fluid from purple to deep blue (blue colour of the dye). This change was only visible in daylight. Therefore this titration of iodine in ethanolic solution where starch was useless as indicator, did not give as good results as an ordinary iodine titration (variations up to 20%).

Action of lysozyme upon stained cells of Bacillus M. Since lysozyme reacts only in solutions containing Na^+ the stained bacterial suspensions were centrifuged and the deposits resuspended in the same volume of Ringer solution. Lysozyme (1 mg./ml. suspension) was then added.

RESULTS

Gram reaction with untreated and lysozyme-treated Bacillus M

Crystal violet uptake. As shown in Fig. 1 treatment with lysozyme before staining decreased the subsequent dye uptake, and the organisms which show a polar separation of cell wall from protoplasm and which stain Gram negative on ordinary smears (Scherrer, 1961) take up less dye than intact organisms. Protoplasts and 'small bacilli' showed the smallest dye uptake. This is true whether dye uptake is compared on the basis of equal volumes of suspensions or on the basis of equal dry weight of suspended material.

As shown by Finkelstein & Bartholomew (1953), the dye uptake depends on the ratio available dye:quantity of cells. Here, too, this ratio seems valid. The 1 ml. suspensions of Bacillus M or its lysozyme-treated forms give the largest dye uptake.

The difference between the dye uptake of intact cells and of cell forms with a polar separation of the cell wall from the protoplasm is of interest, for these cell forms stain Gram-negative on ordinary smears. Lysozyme seems to transform the dye-uptake properties of the cell even before it destroys the whole cell wall.

Table 1. *Dry weights of* 1 *ml. suspension of Bacillus* M *and of its lysozyme-treated forms*

Suspensions in deionized water. Dry weight determinations of samples of 30–50 ml. suspensions.

Intact cells	1·20 ± 0·03 mg. (25*)
Forms with polar separation of cell wall from protoplasm, formaldehyde-fixed	1·07 ± 0·03 mg. (3*)
Formaldehyde-fixed protoplasts	0·68 ± 0·04 mg. (12*)
Unfixed 'small bacilli' free from cell walls	0·68 ± 0·04 mg. (12*)

* Number of determinations.

Since intact bacilli take up more dye than protoplasts or 'small bacilli', it may be assumed that the cell wall takes up some dye, if one doesn't admit that the uptake also depends on the integrity of the cell wall. Some experiments made with preparations of cell walls (consisting of about 95 % cell walls and 5 % intact bacteria after Mickle disintegration and several centrifugations at 5000 rev./min.) give a rather high dye uptake, somewhat higher than the difference between the dye uptake of the intact organisms and the protoplasts. Because of the presence of intact organisms and cytoplasmic contamination in these preparations of cell walls, this dye uptake cannot be compared with the other experiments.

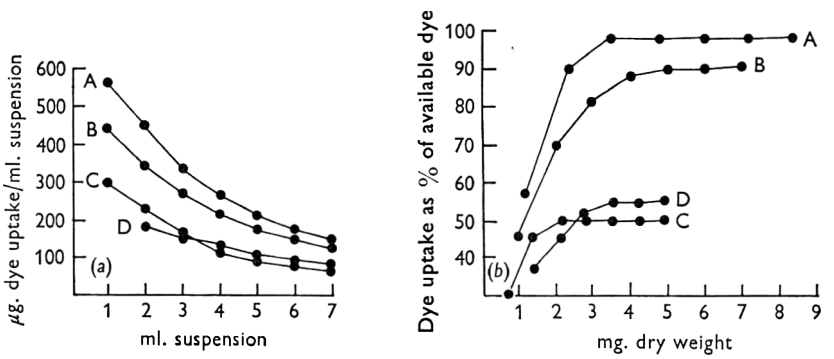

Fig. 1. Crystal violet uptake of Bacillus M. Comparison of the dye uptake of equal numbers (expressed in ml. suspension) or equal dry weights of cells and lysozyme-treated forms. Suspensions in deionized water, pH 6·1–6·3. Total dye available: 1 mg.A, intact Bacillus M cells, unfixed or fixed (70*); B, forms which show a polar separation of the cell wall from the protoplasm (21*); C, fixed protoplasts (14*); D, unfixed 'small bacilli' free from cell walls (21*); *, number of determinations. The curves indicate arithmetic mean values.

Microscopic examinations of wet-mount preparations of intact and crystal violet stained Bacillus M showed evenly coloured bacteria (Pl. 1, figs. 1, 2). No visible segmentation of the bacterial chain was seen at the highest dye uptake level (Pl. 1, fig. 1), but there was some segmentation at lower dye uptakes (Pl. 1, fig. 2). The thickness of the bacillus depends on the dye uptake, as a comparison of figs. 1

and 2 in Pl. 1 will show. The capsule is stained at the highest dye uptakes, for negative staining of crystal violet stained bacteria indicate a slightly blue capsule (Pl. 1, fig. 3), although microscopic examination showed that the Indian ink had extracted much dye from the bacilli. A comparison of the cell dimensions between Pl. 1, fig. 1 and 3 (wet-mount preparation and negative staining) gives the same result. Air-dried smears of crystal violet stained cells showed partially coloured capsules (Pl. 1, fig. 4), but only at the highest dye uptake values. All lysozyme-depolymerized forms showed a deeply blue protoplasm and a slightly blue cell wall (Pl. 1, fig. 5) in wet-mount preparations. Protoplasts and 'small bacilli' were both evenly stained blue.

Effect of washing after crystal violet. Washing the crystal violet stained sediments of the bacilli or the cell forms with deionized water removed a small amount of dye. It was usually not more than 1–5%, except for the 1 m. suspensions where it was

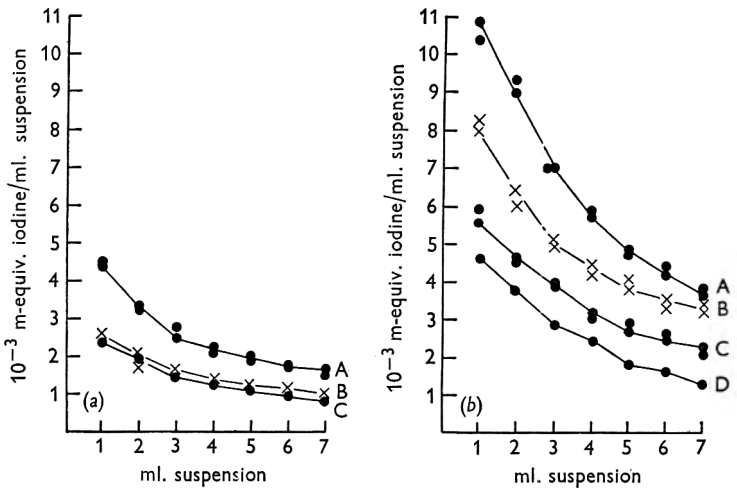

Fig. 2*a*, *b*. Iodine uptake of Bacillus M. Uptake of colourless cells (*a*) and cells stained with crystal violet (*b*). A, Intact Bacillus M (14*); B, forms with a polar separation of the cell wall from the protoplasm (14*); C, fixed protoplasts (14*); D, unfixed 'small bacilli' free from cell walls (7*); *, number of determinations.

higher for the suspensions of intact bacilli. The dye loss was relatively higher for organisms which still possessed an intact cell wall than for all lysozyme-depolymerized forms. Microscopic examination showed that the capsule was no longer coloured after the washing step. No other changes were observed.

Iodine uptake. Wensinck & Boevé (1957) and Scherrer (1963) showed that even colourless organisms take up some iodine. The iodine uptake depended on the ratio iodine:cells. Figure 2*a* shows that the action of lysozyme decreased a subsequent iodine uptake by colourless organisms of Bacillus M; all lysozyme-depolymerized forms took up less iodine than did intact organisms.

Crystal violet stained cells (Wensinck & Boevé, 1957) take up much more iodine because of the formation of a dye iodine complex. Figure 2*b* shows the iodine uptake of intact organisms and treated forms after the crystal violet uptake. Here, too, iodine uptake was highest for intact organisms, lower for forms with a polar separa-

tion of cell wall from protoplasm, and lowest for protoplasts and 'small bacilli'. Extra iodine uptake depends on the previous crystal violet uptake (extra iodine = total iodine uptake less iodine taken up by colourless cells). A plot of extra iodine uptake as a function of net crystal violet content (net content = initial dye uptake less the dye extracted by the washing and iodine treatment steps) gives straight lines (Fig. 3). Therefore, as the proportions of the dye and iodine which intact organisms and lysozyme-depolymerized forms have taken up are the same, the dye iodine complex formed in the intact organism must be the same for all cell structures, cell wall and protoplasm. As there was a difference in the extra iodine uptake as between the intact organisms and protoplasts, the cell wall must take up some extra iodine for the formation of this same dye iodine complex.

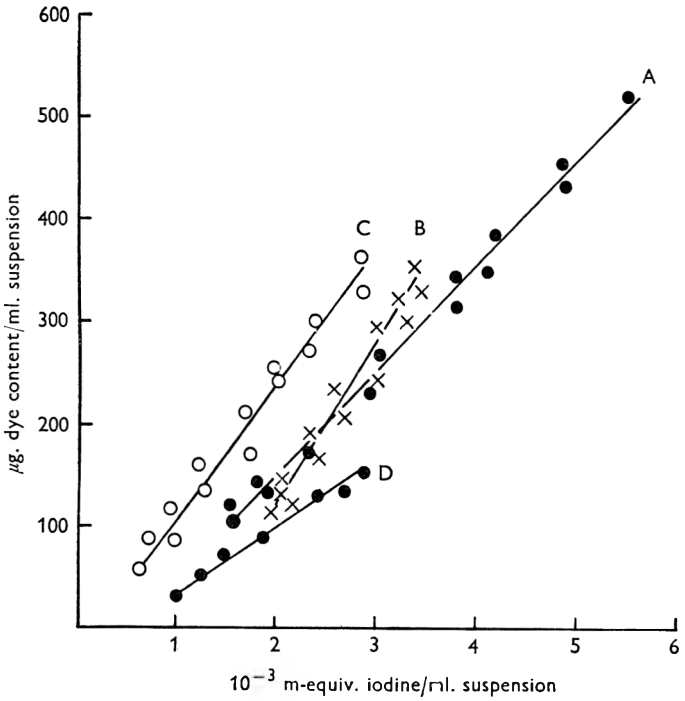

Fig. 3. Extra iodine uptake as in function of the net crystal violet content of Bacillus M. A, Intact Bacillus M (14*); B, forms with polar separation of cell wall from protoplasm (14*); C, fixed protoplasts (14*); D, unfixed 'small bacilli' free of cell walls (7*); *, number of determinations.

A small quantity of crystal violet was extracted by the iodine treatment, especially with bacteria which still possess their cell wall. Microscopic examinations of wet-mount preparations showed small droplet-like formations at the surface of the most deeply coloured organisms after the iodine step (Pl. 1, fig. 6). These formations were deep blue and were also seen at the cell walls of the forms which showed a polar separation of cell wall from protoplasm. Forms free from cell wall, such as protoplasts or 'small bacilli', did not show these droplets. This could lead to the hypothesis that they are due to interactions between dye and iodine at the cell surface, the cell wall or the capsule.

Decolorization. The amount of crystal violet and iodine extracted by 95 % (v/v) ethanol from crystal violet and iodine stained bacilli or cell forms depended on the nature of the material: unfixed Bacillus M suspensions gave up about 20–30 % of their net crystal violet content, whereas formaldehyde-fixed organisms lost 30–45 % of their net dye content (Fig. 4*a*). The total quantity of dye extracted by ethanol was much larger for the cell forms which showed a polar separation of cell wall from protoplasm; it was about 80–90 % of their net dye content. Protoplasts and 'small bacilli' lost about 80 % of their net dye content. Cell wall preparations such as those described on p. 138 lost most, namely 90–95 %.

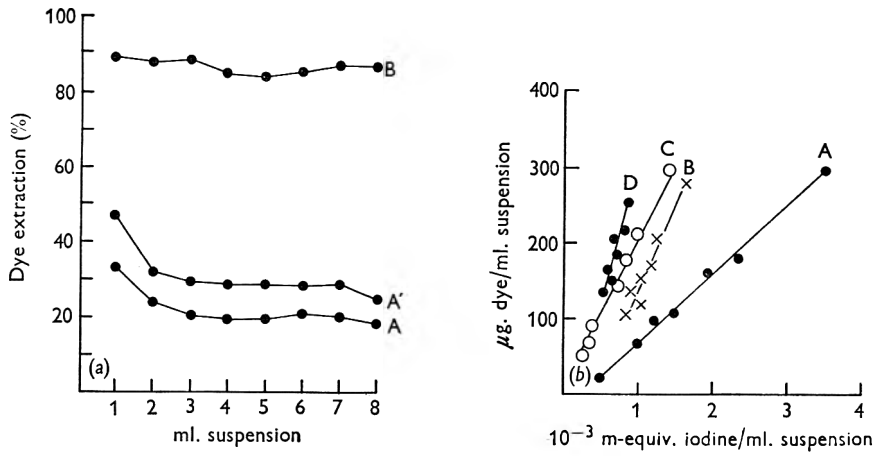

Fig. 4*a*, *b*. Crystal violet and iodine extraction of Bacillus M by 95 % (v/v) ethanol. Dye extraction in % of the net dye content (*a*) and dye extraction as a function of the iodine extraction (*b*). A, Unfixed Bacillus M (39*); A', formaldehyde-fixed Bacillus M (21*); B, formaldehyde-fixed forms with polar separation of cell walls from protoplasm (14*); C, formaldehyde-fixed protoplasts (7*); D, unfixed 'small bacilli' free from cell walls (7*); *, number of determinations. The curves indicate arithmetical mean values. Results of iodine titration in alcoholic solution varied about 20 %.

The dye loss of all lysozyme-depolymerized forms was about twice that of intact bacteria. All these lysozyme-treated forms were Gram negative on ordinary smears. They differed from the intact organisms only by having their cell walls more or less dissolved with lysozyme, which suggests that the partial dissolution of the wall must be responsible for the larger extraction of the dye.

The iodine loss of the decolorization step of this Gram staining depended on the loss of crystal violet (Fig. 4*b*), although even colourless cells lost a very small amount of iodine. The ratio dye loss:iodine loss was the same for intact organisms as for the uptake of dye and extra iodine (Fig. 3). Therefore the dye iodine complex extracted by ethanol must be the same as that originally formed in the cell. Lysozyme-treated forms show other ratios. This may be due to the titration method of the iodine, for it seems unlikely that the extraction transforms this complex when the cell wall is no longer intact.

The colour of the different suspensions after decolorization depends on their dye content, as shown by microscopic and macroscopic examination: intact organisms

were deeply blue while lysozyme-treated forms are white or colourless. Microscopic examination showed that all types of cell were smaller after the decolorization step (see Pl. 1, fig. 7).

The action of lysozyme on Bacillus M *during the different steps of the Gram stain*

The action of lysozyme on crystal violet stained cells. The addition of lysozyme to crystal violet stained organisms in Ringer solution did not give any measurable change in the dye content as compared with the control suspensions, although both lost much dye. The competition of the salt ions of the Ringer solution produces an extraction of the dye (McCalla, 1941). Microscopic examination showed, however, that lysozyme transformed the intact stained bacteria into forms free from cell wall. This transformation was much slower than with colourless bacteria and its result depended on the degree of dye uptake, i.e. only rodlike 'small bacilli' were formed at the highest dye uptake value and protoplasts only at the lowest dye value.

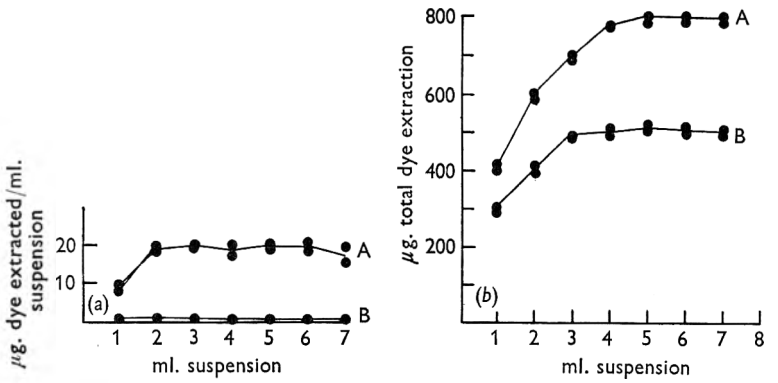

Fig. 5*a*, *b*. Influence of lysozyme on the Gram staining of Bacillus M. (*a*) Action of lysozyme (4 hr.) on cells stained with crystal violet and iodine. Extraction of a small amount of dye. (*b*) Ethanolic extraction of the stained forms (crystal violet and iodine) obtained by the action of lysozyme. A, Bacillus M treated with lysozyme (14*); B, control suspensions of stained Bacillus M (14*); *, number of determinations.

The action of lysozyme on iodine treated cells. The action of lysozyme on organisms which have been suspended in the iodine solution and were then resuspended in Ringer solution was much delayed, but gave the same forms as with crystal violet stained cells; namely, protoplasts at lowest iodine uptake, 'small bacilli' at highest iodine uptake. Iodine is known to inhibit the action of lysozyme (Thompson, 1941).

The action of lysozyme upon crystal violet and iodine stained bacilli. Lysozyme transformed these organisms into rodlike 'small bacilli' at all values of dye uptake. This transformation was very slow and did not release much crystal violet (Fig. 5*a*) The control suspensions did not give measurable amounts of dye. Figure 5*a* shows that the dye loss caused by lysozyme was the same for all cell concentrations used (about 20 μg. dye/ml. suspension), except for the highest dye uptake level where the lysozyme did not entirely dissolve the cell wall (as seen by microscopic control). Iodine was found in all the supernatant fluids.

Therefore the cell wall contained small quantities of dye and iodine that were freed by lysozyme. An ethanol decolorization of the 'small bacilli' produced by

lysozyme after the iodine step extracted a much larger amount of dye than with control suspensions (without lysozyme, Fig. 5*b*). The presence of the cell wall seems to inhibit the ethanol extraction of the dye iodine complex.

The action of lysozyme on Gram-stained organisms. Lysozyme reacted with Gram-stained (crystal violet stained, iodine treated and ethanol extracted) Bacillus M and transformed them into rodlike 'small bacilli' free from cell wall much more quickly than it did after the iodine step of the Gram reaction. Lysozyme did not extract any amount of dye larger than from the control suspensions. Therefore the cell wall no longer contained dye or iodine after the decolorization step. An extra third ethanol extraction of these lysozyme-treated forms released much more dye into the supernatant fluids than in the control. Salton (1961) found the same effects of lysozyme upon Gram-stained organisms.

DISCUSSION

The crystal violet uptake of Bacillus M is changed by the action of lysozyme, for neither comparisons of the same number of intact organisms and all its lysozyme-depolymerized forms such as cells with a polar separation of the cell wall from the protoplasm, protoplasts, or 'small bacilli' free from cell wall, nor comparisons of the same weights of those cells and cell forms indicate the same dye uptake. The dye uptake of all forms is smaller than that of intact organisms. Therefore lysozyme must change the absorptive properties of the bacteria for crystal violet. Lysozyme depolymerizes the capsule and the cell wall of Bacillus M (Tomcsik & Guex-Holzer, 1952). These cell structures, especially the cell wall, may play an important role in the dye absorption by bacteria. It is still under discussion whether Gram-positive and Gram-negative bacteria take up the same quantity of primary dye (Kennedy & Barbaro, 1953; Bartholomew & Finkelstein, 1954; Wensinck & Boevé, 1957; Finkelstein & Bartholomew, 1960). The present experiments show that forms which showed a polar separation of cell wall from protoplasm (Gram negative on ordinary smears) took up less dye than did intact organisms of Bacillus M. This difference in dye uptake is only important for the most dilute bacterial suspensions where the ratio dye:organism is the highest; it is of no interest by comparison with the much larger extraction (decolorization) of dye iodine complex from these lysozyme-depolymerized forms.

Wensinck & Boevé (1957) showed that the iodine uptake depends on the previous dye uptake; dye and iodine form a complex, and this complex is the same in cell wall and in protoplasm. The present experiments indicate that this same complex is extracted from whole organisms by the ethanol decolorization. It may be supposed that the other proportions of dye and iodine which the decolorization step extracts from all lysozyme-depolymerized forms are due to the method of titration.

The dye uptake of the cell wall—that of the capsule does not matter for it is washed out easily—seems rather important after the dye uptake step, but the subsequent wash and iodine steps extract much dye from it.

The cell wall of Bacillus M does not play an important role for the content of the whole organism in dye or iodine, for the dissolution of cell wall by lysozyme after the iodine treatment of the Gram reaction does not free much dye iodine complex. Bartholomew & Finkelstein (1958) showed that the staining of the cell wall region

is not essential for the Gram positivity of fixed and Gram-treated bacteria. It is not the dye or iodine content of the cell wall that is important for the Gram positivity of the whole organism, but its chemical integrity. Partial or total dissolution of the cell wall by lysozyme before the Gram treatment as well as its destruction after the iodine step permit a much larger extraction of dye iodine complex and would give therefore Gram-negative organisms. Wensinck & Boevé (1957) and Salton (1961) pointed out that changes induced by higher ethanol concentrations inhibit extraction of the dye iodine complex from Gram-positive cells. The present experiments have shown that the cell wall is responsible for this inhibition.

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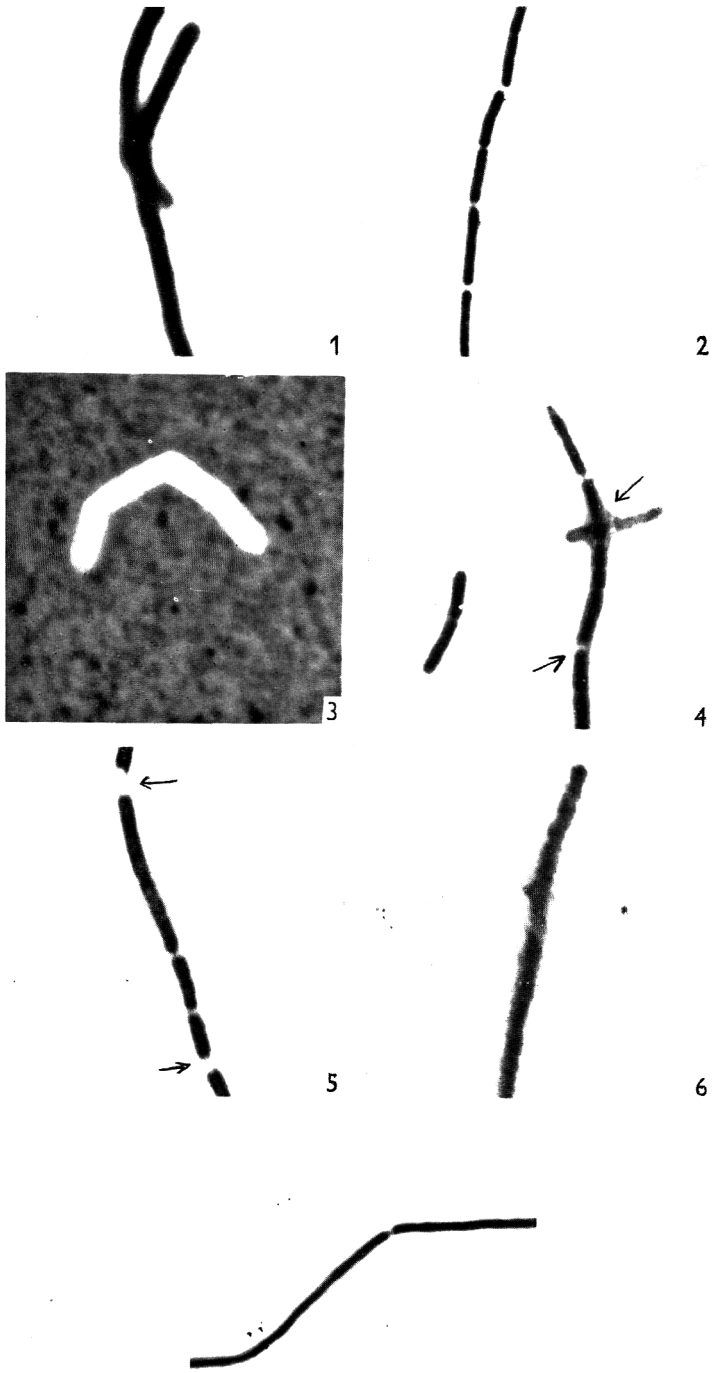

1 2 3 4 5 6 7

R. SCHERRER

(*Facing p.* 144)

EXPLANATION OF PLATE

Gram stain of Bacillus M in suspension. The magnification of all figures is 2000. Figs. 1, 2, 3, 5, 6 and 7 are micrographs of wet mount preparations on slides (1 drop of suspension of stained bacteria was put on a slide, recovered with a coverslip and examined in ordinary microscope with oil-immersion). Fig. 4 is an air-dried preparation (1 drop of the bacterial suspension was put on a slide and examined in the same manner after air-drying).

Figs. 1 to 5. Crystal violet uptake of Bacillus M.

Fig. 1. Cells after the uptake of 550 μg. dye/ml. suspension.

Fig. 2. The same, only after uptake of 200 μg. dye/ml.

Fig. 3. Negative staining of the bacilli in Fig. 1 with Indian ink. Note the capsules.

Fig. 4. The same as in Fig. 1, only air-dried preparation. Capsular staining (arrows).

Fig. 5. Lysozyme-depolymerized forms of Bacillus M with polar separation of cell wall from protoplasm. Forms with the highest value of dye uptake. Arrows: cross walls.

Fig. 6. Bacillus M stained with crystal violet and iodine. Note the dropletlike formations at the cell surface.

Fig. 7. Gram-stained Bacillus M (counterstain omitted).

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With 1 *plate*
Printed in Great Britain

The Habitat of 'Bacterium eurydice'

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(*Received* 20 *September* 1962)

SUMMARY

The alimentary canal of adult bees is the main source of 'Bacterium eurydice' which spreads between bees and to larvae, pollen and honey, from the mouths of infected adults. Most natural spread happens when foraging activity is greatest, probably because infected adults then collect and contaminate pollen which is soon eaten by the young brood-rearing bees.

INTRODUCTION

'Bacterium eurydice' White (*Achromobacter eurydice*, *Bergey's Manual*, 1957) is usually abundant in honey-bee larvae with European foulbrood disease (White, 1920; Bailey, 1960) but its habitat has been givenas 'unknown' (*Bergey's Manual*, 1957). Reports of its presence in apparently healthy bees (Burri, 1947; Gubler, 1954) led us to study its incidence in normal colonies of bees and make observations to find its source.

METHODS

Medium. The following medium was used: glucose 1 g.; yeast extract (Difco) 1 g.; KH_2PO_4 1·36 g.; water 100 ml.; adjusted to pH 6·6 with KOH, autoclaved at 116° for 20 min. and 5 ml. of 25 % (w/v) honey (previously acidified with 0·1 %, v/v, H_3PO_4 and autoclaved) then added. This honey broth was solidified when required with 2 % agar to make honey agar. Unless stated otherwise, inoculated media were incubated anaerobically in McIntosh & Fildes jars at 35°. 'Bacterium eurydice' grows well in these conditions and contaminants were rare.

Bees. The part of the body to be examined was triturated, with sterile water when necessary, and inoculated to honey broth or pour-plates, or streaked on plates of honey agar. The identity of organisms growing in honey broth was examined by streaking on honey agar.

Live adult bees from ten widely separate parts of the British Isles, ranging from Kent and Cornwall to Yorkshire and Northern Ireland, were examined in March and May 1958. The other bees were from the Harpenden locality.

Pollen and honey. Pollen either as pollen-loads, each of about 20 mg. and freshly collected from local foraging bees, or equivalent amounts from stores in combs, was streaked on honey agar plates. Samples (1·0 ml.) of decimal dilutions of honey were inoculated into pour-plates of honey agar.

Flowers. Receptacles of flowers, on which local bees were working, were triturated (without perianths) in water and each inoculated into a pour-plate of honey agar.

RESULTS

Natural incidence of 'Bacterium eurydice'. Figure 1 summarizes results of observations in 1958 and 1959. Local adult bees and those from elsewhere at the same

time of the year, showed the same proportion of infection. Table 1 shows that 'Bacterium eurydice' survived only a few days in stored pollen or honey.

Infection of caged bees in the laboratory. Groups of about 20 adult bees were maintained in small cages at 30°. The average length of life of individuals was about 3 weeks and each was replaced when it died by a bee from a normal colony. Such groups of bees remained infected indefinitely (at least 1 year) provided they were

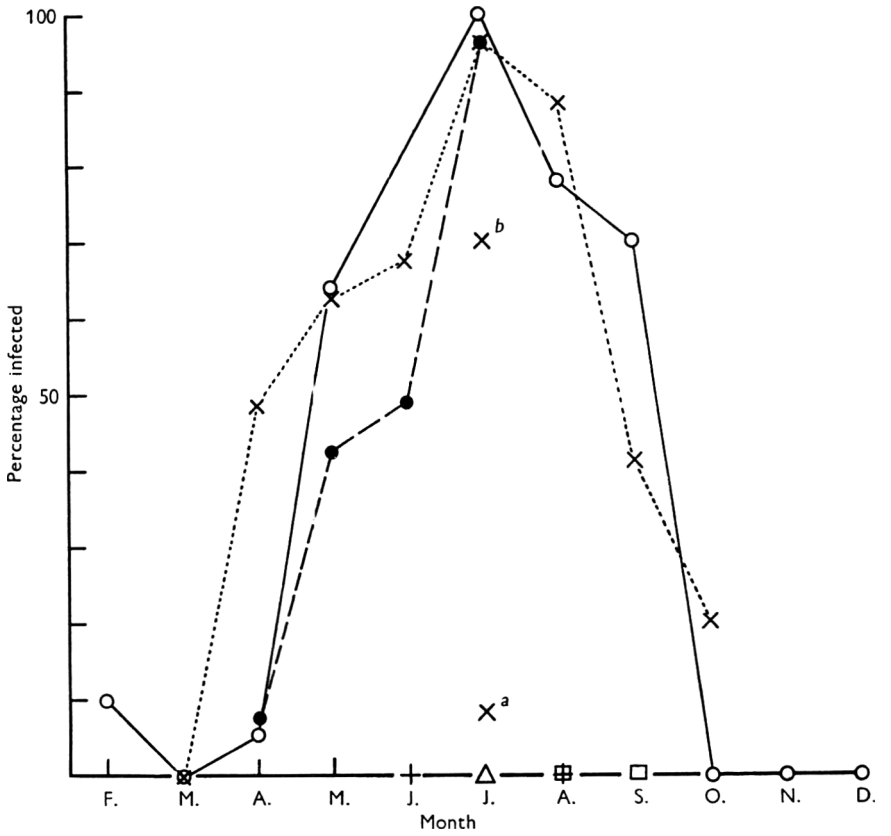

Fig. 1. The percentage of honey-bee adults and larvae, pollen loads and flower receptacles infected with 'Bacterium eurydice'. ○ = adult bees (crops) (average of 26 bees per sample). × = 4- to 5-day-old larvae (mid-guts) (average of 127 larvae per sample). $\times^a$ = 2- to 3-day-old larvae (mid-gut) (30 larvae). $\times^b$ = 3- to 4-day-old larvae (mid-guts) (10 larvae). ● = pollen loads (average of 58 loads/sample). + = rectal contents of infected adult bees (6 bees/sample). □ = newly emerged bees (crops) (12 bees/sample). △ = receptacles of flowers: *Trifolium repens*, *Rubus fructicosus*, *Sonchus oleraceus*, *Chamoenurion angustifolium*, *Lamium album*; 10 of each.

supplied with solid food (a stiff paste of sucrose + water or honey), with water in a separate container. The surface of the food became heavily contaminated with 'Bacterium eurydice', and, according to plate counts, several thousand viable 'B. eurydice' organisms were regularly found in the head, mouthparts and crop of the bees; but this infection dwindled almost to nil within 14 days after the food was changed to sucrose solution or honey.

Newly emerged bees remained uninfected when they were kept separately.

They acquired only slight infection (fewer than 100 bacteria per head) 6 days after they had been put with infected bees in cages containing solid food, whereas they became heavily infected (thousands of bacteria per head) within 6 days when they were separated from the infected bees (and their food) by a wire-gauze screen through which the infected bees fed them liquid food from their mouths. Attempts were made to infect newly emerged bees in cages by feeding them high concentrations of freshly isolated cultures of 'B. eurydice' in liquid food and on solid food, but all failed.

Table 1. *Survival of 'Bacterium eurydice' in pollen and honey freshly stored by bees and subsequently kept at* 18°

Material and date removed from bees	Date	Infection
Pollen, 4 June	4 June	14/21 samples infected
	9 June	9/20 samples infected
	11 June	0/20 samples infected
Honey, 13 July	13 July	200–500 bacteria/ml.
	27 July	0–8 bacteria/ml.

Characters of 'Bacterium eurydice'. Two general types of 'B. eurydice' were observed (Bailey, 1957): type 1 produced dense white colonies on honey agar; type 2 grew in coarsely granular colonies (Pl. 1, figs. 1, 2). Both types, which were isolated from adult and larval bees and pollen, grew well aerobically. Type 1, the commoner, produced gas in deep honey agar, and type 2 did not. Both grew aerobically on ordinary nutrient agar (Oxoid Nutrient Agar no. 2) + glucose or fructose, potato agar and wort agar. Good anaerobic growth with these media needed added glucose + fructose or honey, and even aerobic growth was sometimes feeble or delayed with them unless honey was added. Growth in deep agar, particularly without honey, was characteristically optimal from the subsurface to a few mm. deep. Good growth, either anaerobic or aerobic, occurred in a Seitz-filtered extract of 10 % (w/w) pollen (collected by bees) in water. Cells of both types of 'B. eurydice' were rods of varying length in honey broth or on honey agar (Pl. 1, fig. 4), whereas growth on nutrient agar, potato agar, wort agar and in pollen extract was frequently streptococcal in appearance (Pl. 1, fig. 5). Neither type grew in dilute honey (2·5 %, w/w, buffered to pH 6·5 with potassium phosphate), Apparently Gram positive *in vivo*, 'Bacterium eurydice' was Gram variable *in vitro*.

Bumble-bees. Bacteria that seemed to be types of 'Bacterium eurydice' were isolated from the heads of *Bombus pratorum* and *B. agrorum.* Like those from honey-bees, the anaerobic growth of these bacteria was much stimulated by honey and they grew as rods on honey agar or as streptococci in pollen extract, but their colonial form differed from those of the honey-bee types (Pl. 1, fig. 3).

DISCUSSION

The alimentary canal of adult bees, especially the anterior end, seems to be the only survival place for 'Bacterium eurydice', which does not persist in rectal contents of adult bees (Fig. 1), stored honey or pollen (Table 1), or larval faeces (Bailey, 1959). Newly emerged bees, and flowers recently visited by infected bees, are free from the organisms, so it seems to have no other natural means of survival.

It is transmitted between adults by mouth; and larvae, honey and pollen are probably infected or contaminated by bacteria from the mouths of adults. The nutritional requirements of 'B. eurydice', other than carbohydrate, can be supplied by the bee, probably from the saliva, because large infections were maintained in caged bees given only solid carbohydrate as food and bees discharge saliva on such food to dissolve it (Simpson, 1960). 'B. eurydice' almost disappeared when bees were given liquid food, perhaps because little saliva was then produced. Some, possibly all, of the factors supplied by pollen extract for the *in vitro* growth of 'B. eurydice' may originate in bees, which add fluid from their mouths to pollen as they collect it (Butler, 1949). Natural populations of the bacterium probably increase first in bees collecting pollen and the bacterium is then transmitted to the young brood-rearing bees which consume the pollen and from them to the larvae they feed. Thus, by July, when foraging and brood-rearing reaches its peak, infection becomes general. Pollen collection ends fairly abruptly at the end of July in the district where most of the observations were made. This probably accounts for the decline of infection in bees and larvae after this time. 'B. eurydice' soon dies in the stored pollen; the young bees, which eat it, are less infected than in summer, and old infected bees, which mostly eat honey, contain few bacteria, as they did in the cage experiments. The apparent loss of infectivity of 'B. eurydice' when grown *in vitro* suggests that it is closely adapted to its host. The bacteria in bumble-bees and honey-bees may be host specific: the only likely means of cross-infection would be through flowers, which do not become contaminated.

Thanks are expressed to Mr D. C. Lee for technical assistance.

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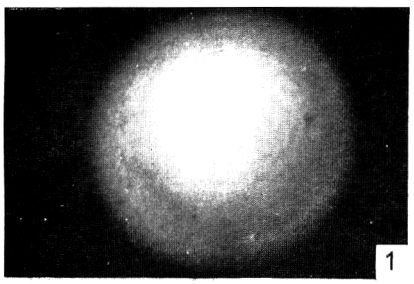
EXPLANATION OF PLATE

Figs. 1, 2. The colonial forms of the two common types of 'Bacterium eurydice' from honey-bees. (× 20.)

Fig. 3. Colonies of 'B. eurydice' from a *Bombus* sp. × 20.

Fig. 4. Cells of 'B. eurydice' grown on honey agar. × 1000.

Fig. 5. Cells of 'B. eurydice' grown in an extract of 10% (w/w), pollen in water. × 1000.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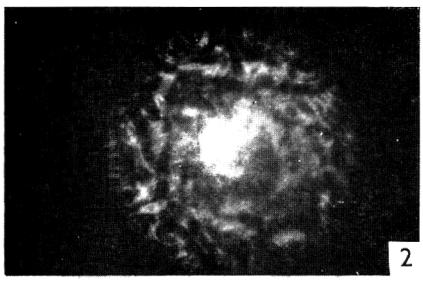
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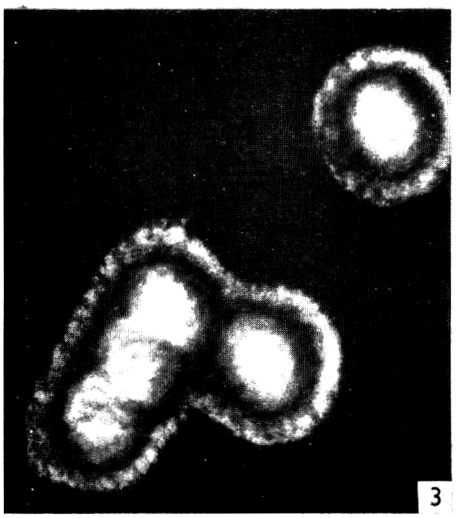
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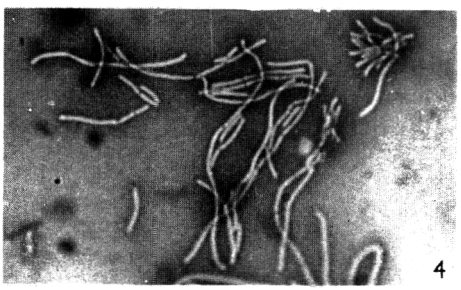
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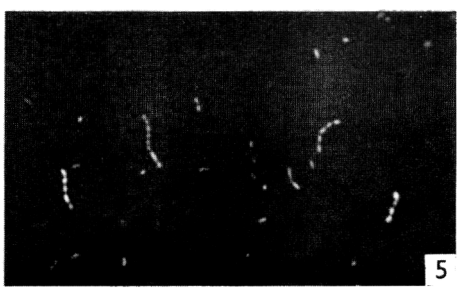
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J. gen. Microbiol. (1963), **31**, 151–160
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On the Osmotic Behaviour of *Saccharomyces cerevisiae* as affected by Biotin Deficiency

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(*Received* 20 *August* 1962)

SUMMARY

The effect of biotin deficiency on the osmotic behaviour of *Saccharomyces cerevisiae* was studied by following the changes in the extinction of dilute suspensions of the yeast in phosphate buffer containing different concentrations of NaCl. The extinction of suspensions of biotin-optimal yeast increased as the NaCl concentration was increased to 1·5 M, but with yeast grown in unsupplemented biotin-deficient medium the readings decreased with increasing NaCl concentration. Yeast grown in biotin-deficient media supplemented with aspartate, or with oleate + aspartate, showed the same type of osmotic behaviour as yeast grown in unsupplemented biotin-deficient medium. But with yeast grown in biotin-deficient medium supplemented with oleate alone, the extinction changes in buffer containing up to 0·7 M-NaCl resembled those of suspensions of biotin-optimal yeast. These differences in osmotic behaviour were not due to breakage of the osmotic barrier by the osmotic pressure differences across the cytoplasmic membrane, but to an increased permeability of the membrane to Cl^-. This was accompanied by an increased permeability to $H_2PO_4^-$ and to higher molecular weight solutes, including bovine plasma albumin. The increase in permeability of biotin-deficient yeast was not as great as that of biotin-optimal yeast in which the osmotic barrier had been broken with 5 % (v/v) *n*-butanol.

INTRODUCTION

The principal known metabolic role of biotin is in the transfer of carbon dioxide in certain carboxylation and transcarboxylation reactions (Lynen, Knappe, Lorch, Jütting & Ringelmann, 1959; Ochoa & Kaziro, 1961). Since the process of CO_2 transfer is of fundamental importance in the metabolism of living cells, any impairment in this process will bring about a major disturbance in cell metabolism. One effect of biotin deficiency in micro-organisms is an impairment in the metabolic processes leading to synthesis of nucleic acids and protein (Ahmad, Rose & Garg, 1961), adenosine triphosphate (ATP; Katsuki, 1959*a*, *b*) and nicotinamide adenine nucleotides (Rose, 1960*a*, *b*). These effects would appear to be due largely to the decreased synthesis of oxaloacetate and aspartate under conditions of biotin deficiency (Shive & Rogers, 1947; Stokes, Larsen & Gunness, 1947) since, with *Saccharomyces cerevisiae*, they can be annulled by growing the yeast in a biotin-deficient medium containing aspartic acid (Ahmad & Rose, 1962*a*). Biotin is also essential for the synthesis of fatty acids through its role in the carboxylation of acetyl-CoA (Wakil, 1961); this explains the biotin-sparing action of certain un-

saturated fatty acids for growth of micro-organisms (Williams & Fieger, 1946; Ahmad & Rose, 1962*a*).

Membranes in micro-organisms are composed predominantly of lipoprotein (Gilby, Few & McQuillen, 1958; Weibull & Bergström, 1958). Since biotin is essential for the synthesis of both major components of these membranes, it is conceivable that membrane structure may be altered in biotin-deficient organisms. The cytoplasmic membrane constitutes the osmotic barrier in micro-organisms, and it follows that any alteration in membrane structure might result in changes in osmotic behaviour. The present paper reports studies on the effect of biotin deficiency on the osmotic behaviour of a strain of *Saccharomyces cerevisiae*.

METHODS

Organism. The strain of *Saccharomyces cerevisiae* (Fleischmann) used was that described previously (Ahmad *et al.* 1961). It was maintained on slopes of malt wort agar: 10 % (w/v) spray-dried malt extract ('Muntona', Munton and Fison Ltd., Stowmarket, Suffolk) + 2 % (w/v) agar. Cultures were stored at 3°.

Experimental cultures. The glucose + salts + vitamins medium (pH 4·5) of Rose & Nickerson (1956) was used. Portions (100 ml.) of medium, containing either an optimal ($8{\cdot}0 \times 10^{-10}$ M) or a suboptimal ($0{\cdot}4 \times 10^{-10}$ M) concentration of D-biotin were dispensed into 350 ml. conical flasks. In some experiments, biotin-deficient medium was supplemented with L-aspartic acid ($2{\cdot}0 \times 10^{-3}$ M) and/or oleic acid (100 μg./ml.). These biotin-sparing compounds were shown to be free from biotin by using the screening procedure described by Ahmad & Rose (1962*a*). Gas chromatographic analysis of the sample of oleic acid used, which was that used in previous studies (Ahmad & Rose, 1962*a*), showed it to contain 76 % (w/v) oleic acid, the principal contaminant being the *trans* isomer of oleic acid, elaidic acid (22 %, w/v). Oleic acid when used in this paper refers therefore to this mixture. Flasks containing medium were plugged and sterilized by autoclaving momentarily at 115°. An inoculum was prepared by suspending sufficient material from a slope culture of the yeast into 6 ml. M/15 KH_2PO_4 (pH 4·5) to give the equivalent of 0·32–0·38 mg. dry wt. yeast/ml. (Rose, 1960*b*). The organisms in this suspension were washed three times with successive portions of M/15 KH_2PO_4 and suspended in 6 ml. of this buffer. Two drops of the washed suspension were added to each flask. Cultures were incubated statically at 25°. Growth was measured turbidimetrically as described by Ahmad *et al.* (1961), extinction readings being related to dry weight of yeast by a calibration curve.

Yeast suspensions. After growth had been measured, the yeast was separated from culture fluid by centrifugation and the crops washed three times in 0·1 M-acetate buffer (pH 4·5; Walpole, 1914) or, when the suspension was to be used for extinction studies, in 0·01 M-phosphate buffer (pH 6·8; Gomori, 1955). The extinction of dilute suspensions of the yeast was measured in 1 cm. glass cells in the Hilger 'Spekker' absorptiometer (Model H 760) with neutral green-grey filters and a water blank.

Thick suspension technique. Chloride-, phosphate-, and protein-permeable volumes of the yeast were determined by using the thick suspension technique described by Conway & Downey (1950) and Mitchell & Moyle (1956). Portions of

washed yeast sufficient to give a packed cell volume of about 1 ml. were centrifuged to constant volume at about 8000*g* in 10 ml. graduated tapered centrifuge tubes which had been calibrated; this took about 40 min. The packed cell volume in each centrifuge tube was noted and the supernatant fluid discarded. By using a fine glass rod the pad of packed organisms was quickly suspended in a standard solution of NaCl (0·10 M), KH_2PO_4 (0·15 M) or crystalline bovine plasma albumin (L. Light and Co. Ltd., Colnbrook, Herts; 0·15 %, w/v) in acetate buffer, exactly equal to the volume of packed organisms. The suspensions were then recentrifuged to constant volume. The supernatant fluids were removed and, after being diluted ten times, analysed for dilution of the probing solute as described below. Each determination was carried out in triplicate and included a control tube in which the pad of organisms was suspended in an equal volume of acetate buffer. The results show the space penetrated by the solute expressed as % (v/v) of the total wet volume of the yeast pad.

Analytical methods. Chloride was estimated by titration against 0·01 N-$AgNO_3$ in a total volume of 1 ml. containing dichlorofluorescein as an indicator (Vogel, 1948). Phosphate was estimated by the method of Fiske & SubbaRow (1925) as described by Umbreit, Burris & Stauffer (1957), with KH_2PO_4 (A.R.) as a standard. Phosphate contents of supernatant fluids from suspensions of the yeast are expressed as μg. P/ml. Protein was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951), with crystalline bovine plasma albumin as a standard. Ultraviolet (u.v.)-absorbing substances in supernatant fluids from yeast suspensions were estimated by measuring the extinction at 260 mμ in 1 cm. cuvettes in the S.P. 500 Unicam quartz spectrophotometer (Unicam, Cambridge), with a blank of the appropriate extracting solution.

RESULTS

Effect of osmotic pressure on the extinction of dilute suspensions of biotin-optimal and biotin-deficient yeast

Ørskov (1945) first reported that cells of *Saccharomyces cerevisiae* swell and shrink according to the osmotic pressure of the surrounding fluid. This change in volume can conveniently be observed by measuring the extinction of a dilute suspension of yeast in solutions of different osmotic pressure. Biotin-optimal yeast from 40 hr. cultures was washed twice with 0·01 M-phosphate buffer (pH 6·8) and suspended at a concentration equivalent to about 0·2 mg. dry weight/ml. in phosphate buffer containing different concentrations of various solutes. After standing at room temperature (18–21°) for 1 hr. the extinctions of these suspensions were measured. Preliminary experiments showed that the changes in extinction, expressed as a percentage of the original reading, were greatest in buffer containing NaCl up to 1·5 M (Fig. 1). There was a slight decline in the extinction of the suspension as the NaCl concentration was raised to 0·25 M, but higher concentrations up to 1·5 M caused an increase in the extinction reading. There was no further change in extinction when the NaCl concentration was increased above 1·5 M. The changes in extinction of suspensions of biotin-optimal yeast in different concentrations of NaCl were completely reversible as was shown by removing the yeast from the NaCl solution by centrifugation and resuspending in an equal volume of phosphate buffer.

Treatment with aqueous butanol is known to break the osmotic barrier in many micro-organisms (Mitchell & Moyle, 1956; Ahmad *et al.* 1961). When biotin-optimal yeast from 40 hr. cultures was suspended in a solution of 5% (v/v) *n*-butanol in acetate buffer (pH 4·5) to a concentration equivalent to about 0·2 mg. dry wt. yeast/ml. before being suspended in phosphate buffer containing different NaCl concentrations, the extinction reading of the suspension declined as the NaCl concentration was increased to 1·0 M; an increase in NaCl concentration to 3·0 M did not cause any further change in extinction reading (Fig. 1).

When yeast grown for 120 hr. in medium containing a suboptimal concentration of biotin was suspended in phosphate buffer containing different concentrations of NaCl, the extinction reading of the suspensions gradually declined as the NaCl concentration was raised to 3·0 M (Fig. 2). Butanol-treated biotin-deficient yeast behaved in an almost identical manner (Fig. 2).

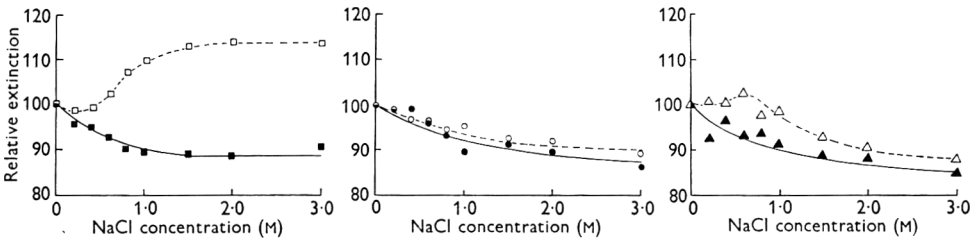

Fig. 1 Fig. 2 Fig. 3

Figs. 1–3. Effect of NaCl concentration on the extinction of dilute suspensions (containing about 0·2 mg. dry wt. equivalent/ml. in 0·01 M phosphate buffer) of yeast grown in a medium containing an optimal concentration (Fig. 1), a suboptimal concentration (Fig. 2) or a suboptimal concentration of biotin + oleic acid (100 μg./ml.; Fig. 3). Open symbols show the behaviour of untreated yeast; filled-in symbols yeast treated with 5% (v/v) *n*-butanol in acetate buffer before suspension in NaCl solutions.

Yeast grown for 120 hr. in biotin-deficient medium containing aspartic acid or aspartic acid + oleic acid when suspended in solutions containing different concentrations of NaCl gave results almost identical with those obtained with yeast grown in unsupplemented biotin-deficient medium. But suspensions of yeast grown in biotin-deficient medium + oleic acid alone showed a slight increase in extinction in the presence of 0·4–0·7 M-NaCl, although higher concentrations caused a decrease in extinction (Fig. 3). The response of yeast grown in oleate-supplemented biotin-deficient medium after treatment with aqueous butanol was similar to that recorded for yeast grown in unsupplemented biotin-deficient medium (Fig. 2). The changes in extinction of the yeast suspensions containing NaCl occurred rapidly on mixing and could be detected after the suspensions had been standing at room temperature for only 5 min. No further change in extinction reading was observed when the suspensions were maintained at room temperature for up to 3 hr.

Effect of biotin deficiency on leakage of cell constituents from yeast suspended in solutions of NaCl

One possible explanation for the differences in behaviour between biotin-deficient yeast and biotin-optimal yeast when suspended in buffer containing NaCl

was that the osmotic barrier of the deficient yeast was broken by the osmotic pressure differences set up across the cytoplasmic membrane. To test this hypothesis, yeast which had been washed twice in acetate buffer was suspended to a concentration equivalent to 5 mg. dry wt./ml. in acetate buffer containing 0·5, 1·0 or 3·0 M-NaCl; control suspensions in NaCl-free acetate buffer were also prepared. The suspensions were allowed to stand at room temperature for 1 hr. and were then centrifuged to remove the yeast. The cell-free supernatant fluids were then analysed for the presence of u.v.-absorbing substances, inorganic phosphate and protein (Table 1).

Table 1. *Effect of* NaCl *concentration on the leakage of ultraviolet-absorbing substances, protein and inorganic phosphate from biotin-optimal and biotin-deficient* Saccharomyces cerevisiae

Yeast grown in media containing an optimal concentration of biotin (BO) or a sub-optimal concentration (BD) with or without L-aspartic acid ($2{\cdot}0 \times 10^{-3}$ M; BD + Asp), oleic acid (100 μg./ml.; BD + OL) or a mixture of these biotin-sparing compounds (BD + Asp + OL) was washed twice with acetate buffer (pH 4·5) and suspended to a concentration of 5 mg. dry wt. equivalent/ml. in acetate buffer either unsupplemented or containing NaCl up to 3·0 M or 5 % (v/v) *n*-butanol. After the suspensions had stood at room temperature for 1 hr., the yeast was removed by centrifugation and the supernatant fluids analysed for u.v.-absorbing substances, protein and inorganic phosphate.

		Contents of supernatant fluids				
		Additions to acetate buffer				
Medium for yeast growth	Age of culture (hr.)	None	0·5 M-NaCl	1·0 M-NaCl	3·0 M-NaCl	5 % butanol
		u.v.-absorbing substances (extinction in 1 cm. cuvette at 260 mμ)				
BO	40	0·05	0·07	0·06	0·09	2·48
BD	120	0·17	0·23	0·25	0·16	7·30
BD + OL	120	0·16	0·21	0·25	0·21	6·60
BD + Asp	120	0·08	0·21	0·27	0·02	1·54
BD + OL + Asp	72	0·09	0·18	0·28	0·17	1·91
		Protein (μg./ml.)				
BO	40	0·0	15·7	9·3	9·3	164
BD	120	0·0	23·6	15·7	11·1	254
BD + OL	120	0·0	2·1	2·1	0·0	286
BD + Asp	120	0·0	17·8	16·3	14·8	97
BD + OL + Asp	72	0·0	10·0	13·7	6·4	154
		Inorganic phosphate (μg. P/ml.)				
BO	40	1·0	3·6	6·0	1·9	20·4
BD	120	0·0	0·0	0·0	0·0	3·6
BD + OL	120	0·0	0·0	0·0	0·0	3·0
BD + Asp	120	0·0	0·0	0·0	0·0	8·2
BD + OL + Asp	72	1·6	2·5	3·0	2·7	11·4

There was a detectable leakage of u.v.-absorbing substances from each type of yeast and this increased slightly in buffer containing NaCl. However, the amounts of these substances in the suspending fluid were small when compared with the amounts liberated in acetate buffer containing 5 % (v/v) *n*-butanol, a treatment which is known to break the osmotic barrier of the yeast cell (Admad *et al.* 1961). The relatively large amounts of u.v.-absorbing substances released from biotin-

deficient yeast grown in the absence of aspartic acid following treatment with aqueous butanol probably include breakdown products of RNA (Rose, 1962). The presence of NaCl in the buffer caused a small leakage of protein although this was not detectable in NaCl-free buffer. Except with biotin-optimal yeast and yeast grown in biotin-deficient medium + aspartic acid + oleic acid, there was no detectable leakage of inorganic phosphate. These results suggested that the slight leakage of cell constituents in buffer containing NaCl up to 3·0 M was not the result of a breakage of the osmotic barrier of the yeast. This was further supported by the observation that the amounts of u.v.-absorbing substances and protein appearing in the extracellular fluid were not in all instances proportional to the concentration of NaCl.

Effect of biotin deficiency on the permeability of yeast to chloride, phosphate and protein

An alternative explanation of the differences in osmotic behaviour between biotin-optimal and biotin-deficient yeast was that the deficient yeast was abnormally permeable to Cl^- so that no appreciable osmotic pressure difference was created across the cytoplasmic membrane. This hypothesis was tested by determining the extent to which Cl^- was able to penetrate yeast grown under various conditions of biotin deficiency, using the thick suspension technique. The results of these experiments are given in Table 2. The value for the chloride-permeable volume obtained

Table 2. *Effect of biotin deficiency on the chloride-, phosphate- and protein-permeable volumes of* Saccharomyces cerevisiae

Yeast grown in media containing an optimal concentration (BO) or a suboptimal concentration (BD) of biotin with or without L-aspartic acid ($2{\cdot}0 \times 10^{-3}$ M; BD + Asp), oleic acid (100 μg./ml.; BD + OL) or a mixture of these biotin-sparing compounds (BD + OL + Asp) was washed three times in acetate buffer (pH 4·5). Wet packed yeast (total volume about 1 ml.) was resuspended in an exactly equal volume of acetate buffer containing NaCl, KH_2PO_4 or crystalline bovine plasma albumin. The suspensions were recentrifuged, and the supernatant fluids analysed for dilution of Cl^-, $H_2PO_4^-$ or protein. The figures quoted are the space penetrated by the probing solute as % (v/v) of the total volume of the yeast pad. Each figure represents the average of at least three determinations.

		Yeast					
Probing solute	Concn.	BO	BD	BD + OL	BD + Asp	BD + OL + Asp	BO treated with 5 % *n*-butanol
		%, v/v, volume of yeast pad					
NaCl	0·10 M	31·4	44·5	37·5	50·9	48·2	87·2
KH_2PO_4	0·15 M	32·0	47·4	34·2	48·1	45·8	88·5
Bovine plasma albumin	0·15 % (w/v)	33·9	58·2	42·8	71·4	74·2	92·0

for biotin-optimal yeast agrees with that reported by Conway & Downey (1950) and is accounted for by the intercellular space between close-packed spheres (about 26 %) and an outer region of the yeast cell which is equivalent to the space occupied

by the cell wall (Conway & Downey, 1950). The chloride ion is incapable of penetrating the cytoplasmic membrane in biotin-optimal yeast. It is, however, able to penetrate the membrane in yeast grown in unsupplemented biotin-deficient medium (Table 2), although the chloride-penetrable volume of this yeast is still well below that of biotin-optimal yeast made completely permeable to Cl^- by treatment with aqueous butanol. Yeast grown in biotin-deficient medium supplemented with aspartate or aspartate + oleate was slightly more permeable to Cl^- than yeast grown in unsupplemented biotin-deficient medium. But the value obtained for yeast grown in biotin-deficient medium supplemented with only oleic acid shows that, although this was slightly greater than the value for biotin-optimal yeast, it was well below that for other biotin-deficient yeasts.

The data reported by Ahmad & Rose (1962*a*) showed that one possible reason for the inability of biotin-deficient yeast to grow as well as biotin-optimal yeast in media containing the biotin-sparing compounds aspartic acid + oleic acid was the apparent inability of this yeast to accumulate phosphate from the medium. In view of the marked differences in permeability to Cl^- as between biotin-optimal and biotin-deficient yeast, it was of interest to examine the effect of biotin deficiency on the phosphate permeability of the yeast. The data in Table 2 show that the changes in permeability to $H_2PO_4^-$ brought about by biotin deficiency were closely similar to the changes in permeability to Cl^-.

The increased permeability shown by biotin-deficient yeast extended to higher molecular weight solutes. The data in Table 2 give the results obtained with crystalline bovine plasma albumin, which had the highest molecular weight of the solutes tested (minimum mol. wt. 69,000; Scatchard, Batchelder & Brown, 1946), and show that biotin deficiency had qualitatively the same effect on the permeability of the yeast to this solute as to Cl^- and $H_2PO_4^-$. Although the small increase in temperature during centrifugation of the thick suspensions did not affect the chloride- and phosphate-permeable volumes of the yeast, it was necessary to use refrigeration (0°) during centrifugation of thick suspensions of the yeast in bovine plasma albumin; otherwise the values obtained for the protein-permeable volumes were extremely high and sometimes exceeded 100 %. The protein is presumably adsorbed on to the yeast cell, the adsorption being greater at higher temperatures. Surface adsorption could explain the somewhat higher values reported for the protein-permeable volumes of the yeast as compared with the corresponding chloride- and phosphate-permeable volume (Table 2).

DISCUSSION

The inability of yeast grown in unsupplemented biotin-deficient medium to show the same type of response as biotin-optimal yeast to changes in the NaCl concentration of the suspending fluid is probably attributable largely, if not entirely, to the increased permeability of the deficient yeast to Cl^-, with the result that no appreciable osmotic pressure difference is set up across the cytoplasmic membrane. This increased permeability of biotin-deficient yeast extends to other low molecular weight solutes (e.g. $H_2PO_4^-$) and also to solutes of molecular weight of the order of that of bovine plasma albumin (about 69,000). But biotin-deficient yeast is not completely permeable to these solutes, possibly because the membranes surrounding

certain organelles in the yeast are not as freely permeable as the cytoplasmic membrane. There are a few reports associating biotin deficiency with altered permeability. Traub & Lichstein (1956) suggested that the biotin-sparing action of oleic acid is due to its ability to allow the more efficient entry of biotin into *Lactobacillus arabinosus*. More recently, Shiio, Ôtsuka & Takahashi (1962) claimed that the copious excretion of glutamic acid by biotin-deficient *Brevibacterium flavum* is due largely to the increased permeability of the cytoplasmic membrane in the biotin-deficient bacterium. Neither of these reports, however, comments on the extent of the permeability changes caused by biotin deficiency or on the nature of the metabolic lesions leading to these changes.

With the data available, it is only possible to speculate about the reason for the increased permeability of yeast grown in unsupplemented biotin-deficient medium. A possible clue is provided by the close similarity in response to changes in the NaCl concentration of the suspending fluid shown by yeast grown in unsupplemented biotin-deficient medium and butanol-treated biotin-optimal yeast. Aqueous butanol is thought to break the osmotic barrier of cells by extracting certain lipid constituents of the cytoplasmic membrane (Hunter, 1961). The cytoplasmic membrane in biotin-deficient yeast may therefore be abnormally permeable because of a deficiency of certain lipid constituents; biotin is known to be concerned in the synthesis of fatty acids (Wakil, 1961). This hypothesis is supported by the finding that the osmotic behaviour of yeast grown in biotin-deficient medium + oleic acid closely approximated to that shown by biotin-optimal yeast. Also the chloride- and phosphate-permeable volumes of yeast grown in biotin-deficient medium + oleic acid approached the values obtained with biotin-optimal yeast. Thus it would seem that the biotin-sparing action of oleic acid and other fatty acids (Williams & Fieger, 1946; Ahmad & Rose, 1962*a*) is based at least in part on the ability of these acids to become incorporated into the lipids of the cytoplasmic membrane.

The diminished synthesis of nucleic acids and protein caused by growing the yeast in unsupplemented biotin-deficient medium is restored when the yeast is grown in a biotin-deficient medium + aspartate (Ahmad & Rose, 1962*a*). But, although protein is a major constituent of cytoplasmic membranes, this restoration of protein synthesis does not restore the permeability properties of the yeast. Instead, it renders the yeast slightly more permeable to small and large molecular weight solutes. Ahmad & Rose (1962*a*) reported that growth of the yeast in a biotin-deficient medium + aspartate + oleate was greater than in biotin-deficient medium supplemented with either of these biotin-sparing compounds alone. Moreover, there was, in this medium, a partial restoration of the lag and exponential phases of growth which characterize growth of the yeast in biotin-optimal medium. But yeast grown in this medium is still abnormally permeable to low and high molecular weight solutes, the ability of oleate to restore the permeability properties of the organism apparently being nullified when aspartate is included in the medium. This may be because one or more of the contaminating fatty acids in the sample of oleic acid used in this study is essential for the restoration of membrane structure and, following the increased growth in this medium, the concentration of this fatty acid in the medium may have become limiting. It is interesting to note too that the activities of certain enzymes in yeast grown in biotin-deficient medium supplemented with aspartate + oleate resemble those of yeast grown in biotin-

deficient medium supplemented with asparate alone, rather than those in yeast grown in biotin-deficient medium containing only oleate (Ahmad & Rose, 1961*b*).

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A Study by Fluorescence Microscopy of the Replication of Lymphogranuloma venereum Virus in HeLa Cell Monolayers

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SUMMARY

In HeLa cells infected with a single infectious unit of lymphogranuloma venereum virus and stained with acridine orange, one particle (initial body) of ribonucleic acid (RNA) about 2μ diam. was seen by fluorescence microscopy in the cell cytoplasm after incubation for 6–8 hr. at 37°. After 11–12 hr. of incubation, an average of 2 particles/infected cell was found. Thereafter the number increased exponentially with a mean generation time of 2–2$\frac{1}{4}$ hr. The particles then remained discrete and in a circumscribed area in the cytoplasm until 18–21 hr., when a vacuole was formed around the initial bodies. By 21–24 hr. smaller particles ranging in diameter from 1μ to about 0·25μ, and in colour from the orange fluorescence characteristic of RNA to yellowish green, were detected amongst the initial bodies. At 33 hr., the initial bodies were almost entirely replaced by smaller particles or elementary bodies, most of which stained yellowish green and had the green fluorescence of deoxyribonucleic acid (DNA) only after treatment with ribonuclease. The elementary body of lymphogranuloma venereum thus consists of a DNA particle surrounded by a layer containing a detectable amount of RNA. After 33 hr. the number of elementary bodies decreased; presumably because infective virus had been released, since at 40–44 hr. initial bodies appeared in previously uninfected cells. HeLa cells infected with more than one infectious unit often contained more than one focus of infection, indicating that replication is not confined to a single site in the cytoplasm.

INTRODUCTION

During the replication of inclusion blennorrhoea virus in HeLa cells, the formation of ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) particles can be observed by fluorescence microscopy (Furness, Henderson, Csonka & Fraser, 1962). In the present investigation multiplication of lymphogranuloma venereum virus in HeLa cell monolayers was studied by the same technique and was found to differ in some respects from that of inclusion blennorrhoea virus.

METHODS

Virus. The JH strain of lymphogranuloma venereum virus (LGV) was grown in HeLa cell monolayers in 250 ml. Pyrex feeding bottles. Suspensions were prepared by dispersing monolayers in 10 ml. tissue culture medium 47–52 hr. after infection

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and releasing the virus by exposure to ultrasonic vibrations (Furness & Fraser, 1962). Tissue culture medium consisted of Hanks's balanced saline solution with the addition of 0·25 % (w/v) lactalbumin hydrolysate, 15 % (v/v) human serum, 5 % (v/v) calf serum, 4 % (v/v) of 1·4 % (w/v) NaH_2CO_3 and 100 µg. streptomycin/ml.

Infection of HeLa cell monolayers for staining purposes. Monolayers on coverslips in Leighton tubes (Furness, Graham & Reeve, 1960) were covered with 0·25 ml. tissue culture medium and inoculated with 0·1 ml. of dilutions of virus suspension; after adsorption for 2 hr. at 30° they were freed from unadsorbed virus by washing with phosphate-buffered saline (Dulbecco & Vogt, 1954), covered with 2 ml. tissue culture medium and incubated at 37°. Sample monolayers were removed at 2–3 hr. intervals for staining. To ascertain the number of infectious units adsorbed, monolayers were stained with Giemsa after 48 hr. incubation and the inclusions counted at a magnification of ×360 (Furness *et al.* 1960). Since the monolayers contained about $1·3 \times 10^6$ cells, the adsorbed virus/cell ratio could be calculated. It was assumed that single infections had been induced in those monolayers with less than 5 % infected cells.

Specificity of acridine orange staining. Acridine orange stained RNA orange and DNA green by our technique (Furness *et al.* 1962). Incubation of uninfected and infected monolayers for 1 hr. at 37° in 0·01 % (w/v) ribonuclease (RNAase) grade A, in McIlvaine's buffer (pH 6·8) and in 0·01 % (w/v) deoxyribonuclease (DNAase) grade B (California Corporation for Biochemical Research, Los Angeles) in veronal buffer (pH 7·4) destroyed the capacity of cell and viral RNA and DNA to stain characteristically with acridine orange, thus confirming the specificity of the staining reactions (Furness *et al.* 1962).

RESULTS

Cells infected with a single infectious unit. The following growth cycle was observed in HeLa cells growing under optimum conditions and infected with a single infectious unit of LGV. After 6–8 hr. incubation at 37°, a relatively large RNA particle, 2µ diam. (initial body) was seen in the cell cytoplasm, usually situated away from the nucleus. By 11–12 hr., an average of 2 particles/cell was counted; their number then increased exponentially for at least 18 hr. (Pl. 1, fig. 1, 2) with a mean generation time of 2–2¼ hr. (Fig. 1). Thereafter they continued to multiply but were too numerous to count, although remaining discrete (Pl. 1, fig. 3). The particles remained in a circumscribed area, and at 18–21 hr. were seen within a vacuole which later developed into the characteristic inclusion body. There was no evidence of an enclosing membrane or matrix. Until this time the particles were relatively uniform in size and it was not until 21–24 hr. that smaller RNA particles were detected in the inclusions. Thereafter the initial bodies gradually decreased in number and were replaced by smaller particles varying in size from 1µ to about 0·25µ and in colour from the typical orange of RNA to yellowish green (Pl. 1, fig. 4). By 33–36 hr. the contents of the inclusions consisted almost entirely of small yellowish green particles with a few irregular RNA masses larger than the initial bodies. At this time the inclusions contained noticeably fewer particles, suggesting release of virus, and this was confirmed by the detection at 40–44 hr. of initial bodies in previously uninfected cells (Pl. 1, fig. 5). We estimated that these

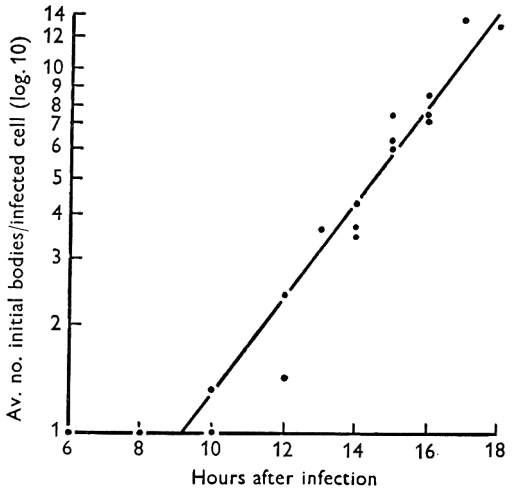


Fig. 1. Multiplication of initial bodies of LGV in HeLa cells infected with a single infectious unit. The points represent the results of four separate experiments.

Table 1. *Distribution of initial bodies in HeLa cells infected with several infectious units* of lymphogranuloma venereum virus*

Cells	Distribution of initial bodies			Initial bodies
No.	No./infected centre			No.
(*a*)	(*b*)			(*a* × *b*)
	One infected centre/cell			
32	1			32
20	2			40
10	3			30
4	4			16
2	5			10
	Two infected centres/cell			
10	1	1		20
5	1	2		15
2	1	3		8
1	1	4		5
4	2	2		16
4	2	3		20
1	3	3		6
1	5	2		7
	Three infected centres/cell			
1	1	1	2	4
1	1	1	3	5
2	1	2	3	12
Total no. cells	Total no. infected centres/100 cells			Total no. initial bodies
100	136			246

* From titration of original suspension, estimated number of infectious units adsorbed 2·5/cell.

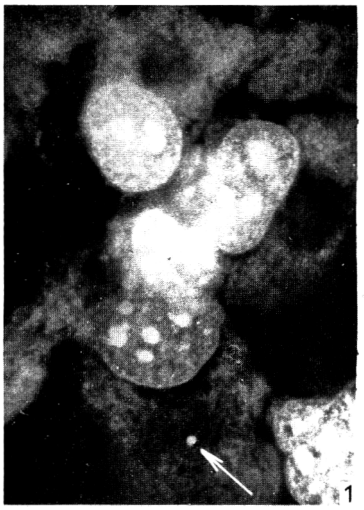
cells had been infected for 12–14 hr., a period compatible with release of virus from the inclusion about 30–34 hr. after adsorption. Since at this time the yellowish green particles are predominant, it is reasonable to infer that they are the infectious units.

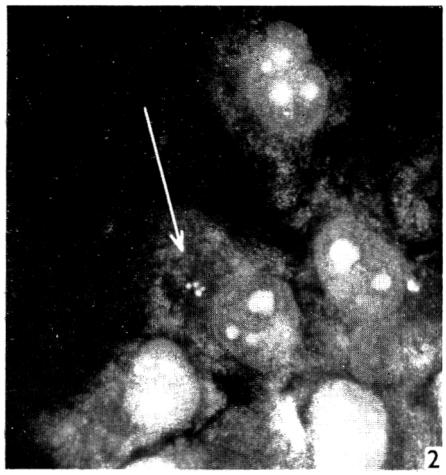
Cells infected with several infectious units. At 9 hr. after infection, cells estimated to have adsorbed an average of 2·5 infectious units contained about this number of initial bodies (Table 1). Of those estimated to have adsorbed 1/10 this number, 95 % of infected cells contained one RNA particle and 5 % two RNA particles. This confirmed that the RNA particles had not yet multiplied (Fig. 1) and suggested that each infectious unit was the precursor of a single initial body. In cells infected with an average of 2·5 infectious units, the number of sites of infection/cell and of initial bodies/site varied. Of 100 infected cells examined, 68 % contained one infected site and 32 % two or more infected sites. Although the virus readily initiated several foci of infection, which usually coalesced to form a single inclusion, many infected centres contained several initial bodies at the outset, which suggests that there is a preferential site for replication.

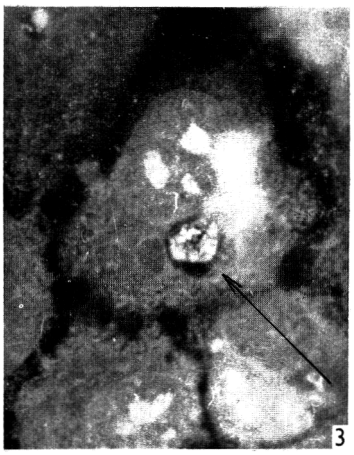
The effect of RNAase and DNAase. Although treatment of the infected cells with DNAase abolished the specific colour reaction of the DNA in the cell nuclei, it did not affect the staining reaction of either the initial bodies or the elementary bodies. Treatment with RNAase abolished the orange and yellow staining properties of the initial bodies and elementary bodies respectively, revealing a core of material that stained green like DNA, and that could be digested by DNAase. The orange fluorescence of the initial bodies and the orange/yellow fluorescence of the elementary bodies can be attributed to varying amounts of RNA in the layer surrounding the DNA core.

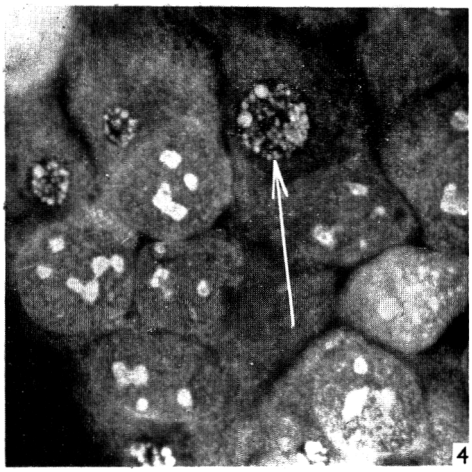
DISCUSSION

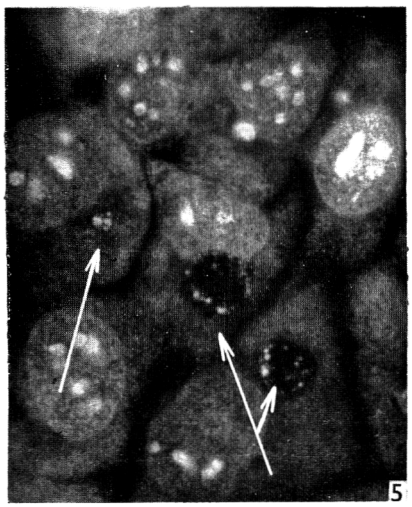
The growth cycles of the trachoma/inclusion blennorrhoea virus (IBV) and the psittacosis/lymphogranuloma venereum virus (LGV) agents are similar. However, the replication of IBV and LGV in singly-infected cells under similar conditions differ significantly. After infection of HeLa cells with either virus, an RNA initial body is derived from each infectious unit. The initial body of IBV is usually found adjoining the cell nucleus (Furness *et al.* 1962), while that of LGV is characteristically away from the nucleus. In cells infected with more than one infectious particle LGV commonly induces several foci of infection, whereas cells infected with IBV have only a single infected centre adjoining the cell nucleus. The initial bodies of LGV stay in a circumscribed area, remain discrete and so can be shown to multiply exponentially for at least part of their cycle. As studied by our routine technique the initial bodies of IBV increase in size without evidence of multiplication, both in singly-infected cells and also in cells infected with several infectious units, where they appear to coalesce forming a large mass of RNA (Furness *et al.* 1962). We have since found that some of these masses of RNA disintegrate into particles similar to the initial bodies of LGV after mounting the infected monolayers, unfixed, in acridine orange stain and compressing the preparation before sealing with beeswax colophonium. This suggests that they are in fact clusters of initial bodies similar to those seen in cells infected with psittacosis virus (Bland & Canti, 1935; Pollard, Starr, Moore & Tanami, 1960).











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Differences in the rate of formation of the inclusions are not distinctive. The appearance of the infectious units or elementary bodies is, however, characteristic. After 18–21 hr. those of IBV are detectable as green-fluorescent DNA particles superimposed on the RNA mass. As the particles increase in number, filling the inclusion, the large masses of RNA gradually disintegrate, finally leaving an occasional RNA particle amongst the DNA particles. By contrast, in LGV at approximately the same time smaller orange to yellow particles are found amongst the initial bodies, which do not form clusters. These particles, which gradually replace the initial bodies, vary in diameter from 1 to 0·25μ, which is the diameter of the DNA particle of IBV, and in colour from orange to yellowish green. The infectious units or elementary bodies stain yellowish green and are without the green fluorescence typical both of DNA and of infective IBV. By treatment with nucleases both viruses can be shown to consist of DNA with an outer layer containing RNA. Thus the differences in staining properties are presumably attributable to a greater quantitity of RNA in the peripheral layer of the elementary bodies of LGV. These results confirm the original observations of Findlay, Mackenzie & MacCallum (1938) and increase our knowledge of the replication of these microorganisms, but also reveal the complexity of their growth cycles, many stages of which remain obscure.

We wish to thank Professor C. F. Barwell for the JH strain of lymphogranuloma venereum virus and Miss Margaret Ham for her excellent technical assistance. The work of one of us (G. W. Csonka) was supported by a U.S. Public Health Service Grant administered by the Medical Research Council Working Party on Nonspecific Urethritis.

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EXPLANATION OF PLATE

Plate 1. Growth cycle of lymphogranuloma venereum virus.

Fig. 1. 8 hr. One initial body.

Fig. 2. 12 hr. Four initial bodies.

Fig. 3. 20 hr. Uncountable number of initial bodies.

Fig. 4. 30 hr. Inclusion body containing both initial and elementary bodies.

Fig. 5. 44 hr. Inclusion bodies with fewer particles and cluster of initial bodies in previously uninfected cell.

J. gen. Microbiol. (1962), **31**, 167–169
With 1 *plate*
Printed in Great Britain

The Occurrence of Struvite (Magnesium Ammonium Phosphate Hexahydrate) in Microbial Cultures

By JANE BEAVON and N. G. HEATLEY

From the Pathological Laboratory, Royal South Hants Hospital, Southampton and the Sir William Dunn School of Pathology, University of Oxford

(*Received* 25 *August* 1962)

SUMMARY

Crystals which occurred within colonies of *Staphylococcus aureus* growing on nutrient agar were identified as struvite (magnesium ammonium phosphate hexahydrate). Other organisms, all of which increased the pH value of the medium during growth, formed similar crystals.

INTRODUCTION

In 1958 one of us (J.B.) noticed that colonies of a particular strain of *Staphylococcus aureus* on plates which had been incubated for 24 hr. and then left in the refrigerator for several days contained well formed birefringent crystals (see Pl. 1). Preliminary investigation encouraged by the late Dr E. S. Duthie, showed that these crystals could be washed repeatedly with water without dissolution, and dissolved only slowly in dilute hydrochloric acid without evolution of gas. They contained phosphorus, and because they blackened on heating it was thought that they were probably organic. The ultraviolet absorption curve was typical of a purine or pyrimidine, the solubility also being characteristic of some of these bases; however, quantitative investigation showed that less than 1 part in 3000 of the crystals could have been purine or pyrimidine. Further tests showed that the crystals were mainly inorganic and liberated ammonia on treatment with alkali in the cold. Magnesium ammonium phosphate is said to be soluble to the extent of 1 part in 7700 of water at room temperature, and comparison with the authentic compound showed that the crystals were almost certainly this substance. Since rigid identification of these small crystals by conventional means would have been difficult, a comparison of them with authentic magnesium ammonium phosphate by X-ray crystallographic methods was sought. Mr H. M. Powell, F.R.S. (Department of Crystallography, University of Oxford) to whom we are deeply indebted for the comparison, reported as follows. 'A single crystal of the first sample provided, a laboratory-prepared specimen of authentic struvite which could not have contained any calcium, was mounted about a principal axis and X-ray diffraction patterns obtained with Cu Kα radiation. Both 15° oscillation and zero layer Weissenberg photographs were taken. The patterns were typical of orthorhombic symmetry for a crystal of cell dimensions approximately $6{\cdot}9 \times 11{\cdot}1 \times 6{\cdot}1$ Å. Nearly one hundred independent reflexions were accessible in the Weissenberg range used; some of those observed were close to the limiting Bragg angle, $\theta = 90°$. Similar Weissenberg

photographs were taken of two crystals obtained from staphylococcal colonies on different [nutrient agar] plates. All three Weissenberg photographs showed, on superposition, complete identity of the spacings of the reflexions, and of the smallest details of the intensities of the different reflexions, including a number which were, for accidental structural reasons, of zero intensity. This constitutes a very firm identification.'

Mr Powell pointed out to us that the occurrence of struvite (magnesium ammonium phosphate hexahydrate) in peptone, tinned food, and material rich in organic matter has been reported at intervals for many years (Groth, 1910; Dana, 1946). Indeed, we later found that Robinson (1889) identified such crystals and suggested '...that the micro-organisms produce the ammonia from the nitrogenous organic matter in which they are growing and that it then combines with the magnesium phosphate present...'. To him undoubtedly belongs the priority in this field.

While we were awaiting the results of the X-ray crystallographic investigation, the paper by Hutchison (1931) on crystals in colonies of *Staphylococcus aureus* appeared; it gives in detail the appearance, properties, conditions under which the crystals are formed. The crystals in his excellent photographs closely resemble our own in appearance, though they were found by him to be a complex calcium phosphate. Dr Hutchison agreed to co-operate with a view to establishing the identity or non-identity of his crystals with our own. However, he has none of his original crystals, and as he points out, crystals prepared by him, now, at least 5 years after his original observations and in a different laboratory, would not necessarily be identical with those originally isolated.

The nature of the colonial crystals might vary with the ions available in the medium derived from different agars, nutrients, or tap water. We investigated this possibility by a number of different experiments, from which we draw the following conclusions. (1) Apparently identical crystals may appear in the nutrient agar as well as, or instead of, in colonies. (2) Both types of crystal are larger and/or more numerous, the larger the amount of Mg added (as sulphate) to the medium. (3) The addition of Ca (as chloride) decreases, or in higher concentration prevents, the formation of extra- or intra-colonial crystals; this is presumably due to removal of some of the phosphate as insoluble calcium phosphate, which can be detected as a slightly increased opalescence in the medium in the higher concentrations used. (4) When nutrient agars of different ionic composition are exposed to ammonia vapour large numbers of crystals form within the agar in a few minutes. They are larger in media which contain added Mg and scantier or absent altogether when extra Ca is present. (5) When the same media is re-exposed to triethylamine vapour, the agar becomes alkaline as quickly as with ammonia, but only after an hour or two are any crystals seen, and they are very scanty or, when Ca has been added, absent. (6) Intra-colonial crystals form more readily in rich media when colonies grow fast to a large size. Presumably the local concentration of ammonia is higher in such colonies than in smaller ones. Ammonia is readily detectable when such plates are opened, both by odour and the effect on litmus paper. (7) Organisms other than the staphylococcus, e.g. *Pseudomonas pyocyanea*, *Proteus* spp. and *Escherichia coli*, will form intra-colonial crystals; within any group those strains which form alkali the more readily are those in which crystals appear earlier and more abundantly. (8) Crystals were also formed by the staphylococcus in fluid

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media, possibly because the organisms tended to sediment and presumably caused a higher local ammonia concentration.

The question of the identity or non-identify of our crystals with those observed by Hutchison thus remains unsettled. The fact that addition of calcium to media suppressed the formation of the typical crystals, by formation of amorphous calcium phosphate, does not rule out the possibility that a crystalline calcium phosphate could be formed under other conditions. On the other hand mere alkalinity, with or without added calcium, leads to very scanty crystals or none, suggesting that ammonium is essential for their formation.

We thank Mr Keith Tyler for taking the photograph (Pl. 1).

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EXPLANATION OF PLATE 1

Colonies of *Staphylococcus aureus* strain 80/52 on nutrient agar. Incubated at 37° for 24 hr. then stored at 4° for 2 weeks.

[*The Editors of the* Journal of General Microbiology *accept no responsibility for the Reports of the Proceedings of the Society. Abstracts of papers are published as received from authors.*]

THE SOCIETY FOR GENERAL MICROBIOLOGY

The Society for General Microbiology held its thirty-fifth General Meeting in the University of Reading, on Thursday, Friday and Saturday, 27, 28 *and* 29 *September* 1962. *The following communications were made:*

SYMPOSIUM: ASPECTS OF CELL-SURFACE ADSORPTION PHENOMENA

Some Sorption Phenomena at Mammalian Cell Surfaces. By L. L. WEISS (*Strangeways Research Laboratory, Cambridge*)

The Mechanism of Influenza Virus Haemagglutination as a Model of Specific Receptor Adsorption. By G. BELYAVIN (*University College Hospital Medical School, London*)

The Bacterial Cell Wall. The Result of Adsorption, Structure or Selective Permeability? By H. J. ROGERS (*National Institute for Medical Research, Mill Hill*)

Some Biologically Important Aspects of Adsorption of Antibody on to Tissue Cells. By J. H. HUMPHREY (*National Institute for Medical Research, Mill Hill*)

Group N Streptococcal Phage Lysin. By B. REITER and J. D. ORAM (*National Institute for Research in Dairying, Reading*)

Abbreviated versions of these contributions will shortly be published in the *Journal of General Microbiology.*

ORIGINAL PAPERS

Some Observations on the Origin of the Charge carried by Bacteria. By G. J. GITTENS and A. M. JAMES (*Department of Chemistry, Chelsea College of Science and Technology, Manresa Road, London, S.W.* 3)

Most bacteria, in suspension at neutral pH values, possess a negative charge. The purpose of this work was to discover whether this charge was due to ionogenic surface groups and/or ion adsorption. The variation of the ζ potential with pH of cells of *Aerobacter aerogenes* and cells treated with ethyleneimine was characteristic of carboxyl- and amino-surfaces respectively. Normal cells treated with diazomethane had zero charge at pH values below 7·5 at all ionic strengths. Surface conductance measurements, on normal and ethyleneimine-treated cells, made over a range of ionic strengths, were used to correct the ζ potentials at the corresponding ionic strengths. The charge density (calculated from the corrected ζ potentials) – ionic strength curves demonstrated a marked difference between the ionogenic surface (i.e. normal and ethyleneimine-treated at pH 4·2) and the non-ionogenic surface (ethyleneimine-treated at pH 10·3). The decrease in charge density of the ionogenic surfaces with ionic strength is due to the adsorption of counter ions on to

the surface ionogenic groups, while the increasing charge density of the non-ionogenic surface is due to the adsorption of anions on to the slightly polar, but uncharged, amino groups.

The results indicate that the charge on normal cells of *Aerobacter aerogenes* is due entirely to the surface carboxyl groups, modified by counter ion adsorption. The adsorption of ions on to the non-ionogenic areas of the surface is possibly inhibited by the highly solvated capsular material and the absence of polar groups on the surface acting as receptor sites. The charge on a bacterial surface may be due to ionogenic groups and/or ion adsorption. The two types of charge may be distinguished from the charge density-ionic strength relationship, after correcting for surface conductance.

Some Observations on the Nature of the Surfaces of Matt and Glossy Variants of Group A Streptococci. By M. J. HILL and A. M. JAMES (*Department of Chemistry, Chelsea College of Science and Technology, Manresa Road, London, S.W.* 3)

The serological difference between the M+ and M− variants of group A streptococci, both of which contain the T antigen, is normally associated with colonial appearance, M+ forming matt and M− glossy colonies. The variation of the hyaluronic acid content of either variant during growth produces a parallel variation of the electrophoretic mobility; whereas hyaluronic acid is readily washed off the surface of young glossy cells, producing a decrease of mobility, it is not removed from matt cells. The T antigen is more readily removed, by tryptic action, from heated matt than from heated glossy cells. Nevertheless, the pH-mobility curves reveal that both surfaces possess similar amino- and carboxyl-groups. Chemical analysis of the M and T protein fractions from both variants of a number of different types showed the presence of 16 and 6 common amino acids respectively, present in the same relative amounts. The surface of the matt variant, however, has more amino- and carboxyl-groups than the glossy, as shown by studies on cells treated with fluorodinitrobenzene.

It is possible that both variants possess the same protein material, but that in the glossy variant this is more highly cross-linked. Thus the matt cells would have a higher density of charge due to the free amino- and carboxyl-groups. The high degree of cross-linking would make the trypsin-sensitive bonds less accessible, thereby accounting for the greater ease of removal of the T antigen from these cells. Further it is probable that the capsular material would bind more strongly to the more highly charged protein of the matt organisms.

A Physical Study of the Capsular and Fimbriative Properties of the Coli-aerogenes Bacteria. By C. F. LIST and A. M. JAMES (*Department of Chemistry, Chelsea College of Science and Technology, Manresa Road, London, S.W.* 3)

Physical techniques have been devised to demonstrate the presence of fimbriae on bacterial surfaces, which may also possess a capsule. Cells of *Klebsiella aerogenes* and *Escherichia coli* when grown in nutrient broth became fimbriate as shown by haemagglutination tests (Duguid, J. P. (1959), *J. gen. Microbiol.* **21**, 271), cells grown on agar were non-fimbriate. The presence of thick or thin fimbriae had no

effect on the electrophoretic mobility of capsular *K. aerogenes*. In contrast the mobility of the non-capsulate variant and also a non-capsulate variant of *Esch. coli* was markedly reduced during growth in nutrient broth, i.e. when the cells became fimbriate. There was also a large difference between the mobility of the capsulate and non-capsulate variants. The coincident pH mobility curves of all the capsulate *K. aerogenes* strains, qualitatively similar to that of the non-capsulate variant, were characteristic of a carboxyl-type surface. Both amino- and carboxyl-groups were present on the surface of *Esch. coli*. In contrast, intrinsic viscosity measurements emphasized the difference between the fimbriate and non-fimbriate forms for the capsular but not for the non-capsular organisms. The intrinsic viscosity of the capsular organisms was twice that of the non-capsular. Haemagglutination tests will always demonstrate the presence of fimbriae; the test is, however, slightly masked by the presence of large capsules. Both mobility and viscosity measurements will distinguish between capsular and non-capsular organisms, independent of the presence of fimbriae. In the absence of a capsule fimbriate strains can be distinguished from non-fimbriate ones by mobility studies, and in the presence of a capsule by viscosity studies. As a result of these tests it was established that the N.C.T.C. culture 418 (*Aerobacter aerogenes*) is capsulate possessing thick-type fimbriae.

The Antigenic Structure of the Reiter Treponeme. By J. H. DE BRUIJN (*National Institute of Public Health, Utrecht, Netherlands*)

The Reiter treponeme is one of the 7 strains which have been cultured from 80 to 90 cases of primary syphilis (Wassermann, A. & Ficker, M. (1922), *Klin. Wschr.* **1**, 1101). By salting-out a lysate of the organisms, the so-called Reiter Protein is obtained which has been successfully introduced as an antigen in the complement-fixation test for syphilis. As a result of serological investigations, this antigen appeared to be a lipo-polysaccharide-protein complex. Generally, only the protein component is responsible for its reactivity with syphilitic sera. The presence in syphilitic serum of an antibody reacting with the protein component of the Reiter treponeme has been ascribed to the occurrence of a 'group specific protein antigen' both in this organism and in virulent *Treponema pallidum*. In the present paper, this group antigen is also demonstrated in *T. zuelzerae*, an anaerobic free-living spirochete, isolated from mud (Veldkamp, H. (1960), *Antonie v. Leeuwenhoek*, **26**, 103). The Reiter treponeme and *T. zuelzerae* turned out to differ in their polysaccharide components.

Discrepant results have been reported in the literature regarding the reactivity of Reiter polysaccharide with syphilitic serum. The present investigations have been carried out with a polysaccharide antigen prepared by the trypsin digestion method (Raistrick, H. & Topley, W. W. C. (1934), *Brit. J. exp. Path.* **15**, 113): 12 out of 205 syphilitic and 3 out of 597 non-syphilitic sera proved to be reactive in the complement-fixation test with this antigen. The low incidence in syphilis, although twelve times higher than in non-syphilitic cases, makes it unlikely that the anti-Reiter polysaccharide antibody is provoked by the syphilitic infection. Although a heterogenetic origin of this antibody cannot be excluded, it seems obvious to suspect the Reiter treponeme as a saprophytic contaminant of the syphilitic lesion of being the causative agent.

The Location of the Group D Antigen in a Strain of *Streptococcus faecalis* var. *liquefaciens*. By P. M. FRANCES SHATTOCK and D. G. SMITH (*Department of Microbiology, University of Reading*)

In group D streptococci the group specific antigen is not part of the cell wall (Elliott, S. D. (1959), *Nature, Lond.*, **184**, 1342; Elliott, S. D. (1960), *J. exp. Med.* **111**, 621; Jones, D. & Shattock, P. M. F. (1960). *J. gen. Microbiol.* **23**, 335). In a further attempt to determine the location of the group D antigen we examined spheroplasts, protoplasts and L-forms derived from group D streptococci for the presence of the group antigen. During the action of egg-white lysozyme (Abrams, A. (1959), *J. biol. Chem.* **234**, 383) on a strain of *Streptococcus faecalis* var. *liquefaciens*, ELV 2025, the group D antigen in the cocci and suspending fluid was assayed by a gel-diffusion precipitin method. During enzymic action about 50 % of the group D antigen was released into solution in 90 min. The resulting bodies appeared not to be true protoplasts but spheroplasts. Protoplasts were produced from the same strain, ELV 2025, by using a phage-associated cell-wall lysin prepared from a phage lysate of *S. faecalis* var. *zymogenes* H69D5 by the method of A. S. Bleiweis & L. N. Zimmerman (1961) (*Canad. J. Microbiol.* **7**, 363). During digestion of the cell wall of the strain ELV 2025 with this enzyme 85 % of the group D antigen was released into solution in 90 min. Similar results were obtained with two other strains of group D streptococci, *S. faecalis* var. *zymogenes*, N37 and D76. The results of these experiments suggest that, at least in these three strains of streptococcus, the group D antigen is not located in the cytoplasm. Further evidence for the non-cytoplasmic location was obtained from a study of L-forms. W. Hijmans (1962, *J. gen. Microbiol.* **28**, 177) found no group D antigen in the L-forms of two strains of *S. faecalis* var. *zymogenes*, 27 and 30, nor in the suspending fluids. We found, however, that after continual subcultivation in the liquid medium of Hijmans, one of these L-forms, 27, produced the group D antigen when transferred to the same medium without penicillin and containing 0·5 % glucose instead of 0·1 %. About 97 % of the group D antigen was free in the culture medium and there was no reversion to streptococcal forms. We conclude that in the strains examined the group D antigen is not located in the cytoplasm. Since we already know that it is not an integral part of the cell wall, we deduce that it is located between the cytoplasmic membrane and the cell wall. A Studentship from the Agricultural Research Council is acknowledged by D.G.S.

The Chemical Composition of the Cell Wall of the Yeast, *Nadsonia elongata*. By K. G. H. DYKE (*Sub-Department of Chemical Microbiology, Department of Biochemistry, University of Cambridge*)

Cell walls isolated from disrupted cells of *Nadsonia elongata* and purified by differential centrifugation are free from significant cytoplasmic contamination. The mean composition of three separate lyophilized preparations was found to be $1{\cdot}92 \times 10^{-4}$ % nucleic acid, 1·70 % nitrogen, 0·03 % phosphorus, 0·4 % hexosamine, 7·3 % lipid, 80 % carbohydrate and 7·8 % protein. The carbohydrate contains 60 % D-glucose and 40 % D-mannose. Hydrolysis of the cell wall with excess 6N-HCl at atmospheric pressure for 24 hr. produced more ammonia than can be accounted for by the breakdown of amides and hexosamines. Since the molar proportion of

ammonia released by 6N-HCl from either cell wall or whole yeast is similar it is assumed that the degree of breakdown of amino acids is similar and that the analyses are comparable. The cell-wall hydrolysate has high molar proportions of threonine and serine and a low molar proportion of lysine. The two hydroxy-amino acids account for nearly 30 % of the amino acid molecules present. Lipid extracted from the cell preparation contains less than 1 % phosphorus. The extracted lipid was saponified, the saponified material esterified with methanolic HCl and the methyl esters separated by gas-liquid chromatography. The material was chromatographed on two stationary phases: a polar phase, polyethyleneglycol adipate and a non-polar phase, Apiezon L grease. The major fatty acids were identified by comparison with standards and found to be hexadecanoic acid, octadecanoic acid and octadecenoic acid. Comparison with the chromatogram obtained for methyl esters derived from the fatty acids of the lipid extracted from whole cells showed that $^{7}\Delta$ hexadecenoic acid which accounted for 60 % of the fatty acid molecules of the whole-cell lipid was completely absent from the saponifiable lipid of the cell wall.

Studies on the Kefir Grain. By J. W. M. LA RIVIÈRE (*Laboratory of Microbiology, Technological University, Delft, Netherlands*)

Kefir milk is prepared by subjecting milk to fermentation by kefir grains, i.e. conglomerates of lactic acid bacteria and yeasts. As asepsis is not maintained during the process, the kefir grain would present a remarkable problem in natural selection if its composition were specific and constant. Since there is no agreement in the literature about the microbial composition, quantitative methods were used in the present work. Counting on different media showed that only 5 % of the viable yeasts fermented lactose; the predominant yeast was *Saccharomyces delbrückii*. The predominant rod-shaped lactic acid bacterium was successfully cultivated on the medium of J. C. de Man, M. Rogosa & M. E. Sharpe ((1960), *J. appl. Bact.* **23**, 130), and was identified as *Lactobacillus brevis*. From these and other experiments it is concluded that the differences in microbial composition earlier found, can be explained by differences in techniques and that the kefir grain is strikingly specific and constant in character. *S. delbrückii* and *L. brevis* are unable to grow in milk by themselves but stimulated development of each other when grown together. This explains how these organisms can grow in kefir milk but does not account for their successful survival among numerous less-exacting microbes. As the material which holds the organisms together in grains must be of high survival value, a chemical analysis was made. This showed that 25 % of the dry weight of the grains consists of extracellular polysaccharide material. On hydrolysis this was found to be composed for at least 80 % of glucose and galactose. Further study of the nature and origin of this material is believed to be essential for comprehension of the process of the formation of the grain itself.

Influence of the Size of Inoculum on Growth of Fungal Cultures. By J. MEYRATH (*Royal College of Science and Technology, Glasgow*)

The influence of the size of inoculum on fungal growth differs from that observed with bacteria, where the size of inoculum affects only the lag phase; the latter was hardly affected in the case of *Aspergillus oryzae*. Several claims in the literature

of effects on the lag phase of moulds are not valid in the author's opinion because this phase has been considered as the time required to obtain measurable growth or a constant rate of growth and this may have implied that up to this point the culture had been growing exponentially. If with *A. oryzae* the size of inoculum is reduced from 8×10^7 to 4×10^3 conidia, or from 12·5 to 0·0008 mg. mycelium (dry weight) per 100 ml., the rate of multiplication in the exponential phase, the rate of growth in the linear phase, and the maximum yield were usually all decreased, although sometimes one or two of these effects were not noted. With high sugar concentration in the substrate and/or poor trace element supply and/or vigorous agitation during cultivation, one or all of these phenomena were increased depending upon whether these factors acted singly or together. Using substrates poor in trace elements it was shown that the trace element content of large inocula was not sufficient to account for any increase of growth over cultures from small inocula. Five other strains of *Aspergillus*, although not tested extensively, showed a similar response to that of *A. oryzae*. Some of a number of yeasts tested also behaved like the moulds (Baird, L. (1962), Thesis, Royal College of Science and Technology, Glasgow). The phenomena reported here are probably not governed by the same mechanism as those observed with *Aspergillus amstelodami* (Darling, W. M. & McArdle, M. (1959), *Trans. Brit. mycol. Soc.* **42**, 235).

Influence of the Size of Inoculum on Some Aspects of Metabolism of *Aspergillus oryzae*. By A. F. McIntosh and J. Meyrath (*Royal College of Science and Technology, Glasgow*)

The influence of the size of inoculum on the growth of *Aspergillus oryzae* appears to be the result of a variation of certain properties of the mycelium during growth. There is a decrease in efficiency of utilization of carbon and nitrogen during cultivation. Therefore, a culture from a small inoculum is characterized by an inefficient mycelium during most of its development. That a small-inoculum culture had a lower carbon utilization efficiency than a large one was shown by the observations made at various stages of growth that more carbohydrate was metabolized in order to produce a given amount of growth, that more ethanol was formed per unit weight of mycelium, and that the specific rate of respiration was smaller. Similarly, the lower efficiency of utilization of the nitrogen source (ammonium sulphate) was indicated by the lower nitrogen content of the mycelium and the larger amount of excreted organic nitrogenous compounds per unit weight of mycelium. The fact that the influence of the size of inoculum was more pronounced when vigorous agitation was used during cultivation suggests that the mycelium was partly disrupted by this. This could be expected to be more pronounced the longer the time of cultivation, i.e. the smaller the inoculum. However, the sensitivity towards this mechanical stress may be restricted to the initial stages of growth, as it was only in these stages that the specific rate of respiration of the mycelium was found to be sensitive to a mild pressing procedure. The above-mentioned phenomena are partly to be explained by the fact that growth-stimulating substances are excreted which, if they act at *early* stages of the culture development, stimulate growth of later stages, and with a large inoculum a certain critical concentration of these substances is established *earlier* than with a small inoculum.

Catalases in *Lactobacillaceae*. By E. A. DELWICHE (*Division of Bacteriology, Cornell University*)

Catalase activity in the lactic acid bacteria, until recently unsuspected and undetected, is demonstrable in certain cultures of *Pediococcus*, *Streptococcus*, *Lactobacillus* and *Leuconostoc*. In recent studies with *Pediococcus* in our laboratory and elsewhere, it was established that the enzyme is developed best when the sugar content of the medium is low, and that the important factor seems to be acid production. The catalase system of *Pediococcus cerevisiae* E66 was studied in detail. The catalytic system in the cell-free state (sonic oscillation) was seen to be heat sensitive, non-dialysable, and not precipitated by centrifugation at 140,000 *g* for 1 hr. It could be destroyed with 8 M urea. Cells grown in the presence of 0·001 M sodium azide were in possession of the enzyme, apparently without diminution, and the cell-free preparations obtained under these conditions, or even in the absence of azide in the growth medium, were insensitive to concentrations of azide and cyanide as high as 0·001 M. It has been concluded that the catalytic principle is probably protein and enzymic in nature. The enzyme could be fractionated with ammonium sulphate (0·70–0·75 saturation), or with acetone (55–66 %). Preparations purified 100-fold were not stimulated by the flavin coenzymes, FAD and FMN, and were insensitive to acriflavin, α-α'-dipyridyl, EDTA, and *para*-chlormercuribenzoate. The system is less sensitive to substrate inactivation than the heme-ion catalases of *Escherichia coli* or beef liver. Detailed studies are being extended to the other members of the Lactobacillaceae.

Effect of Biotin Deficiency on Feedback Mechanisms Controlling the Synthesis of Ornithine Carbamoyl Transferase by *Saccharomyces cerevisiae*. By B. DIXON and A. H. ROSE (*Department of Bacteriology, King's College, University of Durham, Newcastle-upon-Tyne*)

Several micro-organisms show diminished activity of ornithine carbamoyl transferase (EC 2.1.3.3.) when grown under conditions of biotin deficiency (Ahmad, F. & Rose, A. H. (1962*a*), *Arch. Biochem. Biophys.* **97**, 302; Estes, J. M., Ravel, J. M. & Shive, W. (1956), *J. Amer. chem. Soc.* **78**, 6410). Though biotin is known to be a cofactor in certain carboxylase and transcarboxylase reactions, it is not essential for ornithine carbamoyl transferase activity. The object of the present study was to clarify the role of biotin in the synthesis of this enzyme by *Saccharomyces cerevisiae*. The specific activity of the enzyme in yeast grown in unsupplemented biotin-deficient medium was approximately half that of yeast from biotin-optimal cultures. Yeast grown in biotin-deficient medium supplemented with L-aspartic acid showed a marked restoration of nucleic acid and protein synthesis (Ahmad, F. & Rose, A. H. (1962*b*), *J. gen. Microbiol.* **28**, 147), but the specific activity of the enzyme was only one-quarter of that in yeast grown in aspartate-free biotin-deficient medium. The presence of L-ornithine (100 μg./ml.) in the medium approximately doubled the specific activities of biotin-optimal yeast and of yeast grown in unsupplemented biotin-deficient medium, but yeast grown in biotin-deficient medium supplemented with aspartate showed a fivefold increase in specific activity. Although the enzyme was inducible to some degree in yeast grown in these media, end-product repression could not be demonstrated. L-Arginine, up

to 500 μg./ml., in biotin-optimal and biotin-deficient media had little significant effect on synthesis of the enzyme by the yeast in either the presence or absence of ornithine. Also, addition of DL-citrulline, L-proline or L-glutamic acid to biotin-optimal and biotin-deficient media had no detectable effect on enzyme synthesis. In this strain of *S. cerevisiae* it would appear therefore that biotin functions in the synthesis of ornithine carbamoyl transferase partly through its role in the synthesis of inducers (ornithine) and partly through some other mechanism which is independent of the role of biotin in the synthesis of nucleic acids and total protein.

The authors thank Miss J. Hall for valuable technical assistance. One of us (B. D.) is grateful to the Luccock Research Fund for financial assistance.

Glutamic Acid Producing Arthrobacter species. By H. VELDKAMP (*Microbiological Laboratory, Agricultural University, Wageningen, The Netherlands*)

Two hundred and fifty *Arthrobacter* strains, isolated from different soils, were tested for their ability to produce glutamic acid. The strains were kept on yeast-extract glucose agar, and screened in shake flasks using a mineral medium with glucose as carbon source. Of these strains, 25 % produced minor amounts of amino acids, mainly alanine and glutamic acid. Five strains, all of which were biotin dependent, produced considerable amounts of glutamic acid, and one of these was selected for further study. Glutamic acid was only produced by this strain when the biotin concentration of the growth medium was suboptimal for growth. Glutamic acid production was highest in a mineral-glucose medium containing 10^{-5} μg. of biotin/ml.; in this case 0·45 moles of glutamate were produced per mole of glucose utilized. The concentration of $(HN_4)_2SO_4$ in the growth medium appeared to determine the ratio in which glutamic acid and alanine were produced. Excess of $(NH_4)_2SO_4$ resulted in high alanine and low glutamic acid production. The optimal temperature for glutamic acid production was 30–32°. Experiments in which the pH was automatically controlled at 6·0 and 7·0, respectively, showed that at pH 6·0 only glutamic acid was produced; at pH 7·0 less glutamic acid was formed and alanine was always formed in considerable amounts.

Nutritional Studies on Coryneform Bacteria from Soil and Herbage. By J. M. GRAINGER and R. M. KEDDIE (*Department of Microbiology, University of Reading*)

The purpose of this work was to determine and compare the growth factor requirements of coryneform bacteria from soil and grass. A collection of 115 cultures of coryneform morphology (Jensen, H. L. (1952), *Annu. Rev. Microbiol.* **6**, 77) was isolated from one sample each of garden soil (37 isolates), field soil (30 isolates) and grass (48 isolates) using media chosen to avoid selecting for particular nutritional requirements. The isolates were first divided into three groups by methods similar to those of Lochhead & Chase (1943, *Soil Sci.* **55**, 185): B-group comprised cultures which grew in a mineral salts + glucose medium, Y-group cultures required yeast extract while YS-group cultures required soil extract + yeast extract. Soil isolates were represented in all three groups but most (46 isolates) belonged to Y-group. The results for the two soils were similar except that 8 of 9 B-group iso-

lates were from the garden soil. For the grass isolates only one required soil extract; the remainder belonged to Y-group. In all, 8 patterns of growth factor requirements were recorded for the Y-group isolates. Of these, 4 patterns were represented by one strain each and the remaining patterns accounted for a majority of the Y-group isolates. Of the frequently occurring patterns, requirements for biotin + thiamine, or for thiamine alone, were common to isolates from soil and grass. However, a requirement for biotin alone occurred only in soil isolates, while a requirement for biotin + thiamine + pantothenate was found only in grass isolates. A small residue of Y-group strains from both habitats did not grow in a medium containing Casamino acids, biotin, thiamine, pantothenate, nicotinic acid, folic acid and riboflavin. The most frequent pattern among soil isolates of the YS-group was a requirement for vitamin B_{12} + thiamine; this pattern was shared by the single grass isolate in this group. A less common pattern was a requirement for vitamin B_{12} + biotin + thiamine, and one soil isolate required the *terregens* factor + biotin + thiamine. One isolate of this group did not grow in a medium containing yeast extract + vitamin B_{12} + *terregens* factor.

Some Quantitative Aspects of the Gaby & Hadley Test. By N. D. S. Bell and J. Meyrath (*Royal College of Science and Technology, Glasgow*)

The laboratory test for the identification of *Pseudomonas aeruginosa* described by W. L. Gaby & C. Hadley (1957, *J. Bact.* **74**, 356) has been applied in duplicate to 26 strains of *P. aeruginosa* and 30 strains of *P. fluorescens*. The results obtained with the former group varied from 3 to 40 sec. This is in reasonable agreement with the 15–30 sec. reported by Gaby & Hadley. It shows, however, that the test cannot be considered a quantitative one with 15–30 sec. as a criterion of *P. aeruginosa*. The latter group gave results of from 5 to 600 sec. In both groups, the variations between two tests on the same strain could be as large as those between different strains. It seemed possible that interesting quantitative results could be obtained when different species of organism were subjected to the test if sufficiently rigorous standardization of the conditions could be obtained. A strain of *P. fluorescens* was chosen for this investigation. An absorptiometer was used both to standardize the cell suspensions and, using a suitable filter, to record the progress of the reaction. As a simplification, α-naphthol was omitted, the rate of development of the pink colour of oxidized *p*-aminodimethylaniline oxalate being equally suitable for the measurement. The experiments were carried out at 20° in a solution buffered to pH 7·2. Initial velocities of the reagent oxidation were readily obtained by this method but, on trying to establish a linear relation of these to enzyme concentration, it was found that the range of linearity was limited since, on increasing the concentration of the enzyme, the reducing power of the cell material on the substrate became apparent while, on reducing the enzyme concentration, the inhibitory effect which the substrate was found to have on the enzyme also became a greater proportion of the total reaction.

The Neutralizing Action of Saliva and Gastric Juice on Poliomyelitis Virus. By E. Szöllősy and V. Balázs (*Institute of Public Health and Hygiene, Köjál, Szeged, Hungary*)

The neutralizing action of serum, saliva and gastric juice of achlorhydric persons has been tested on all three types of the poliovirus. The gastric juice of such persons will neutralize the cytopathogenic effect of all three on HeLa cells. In general, the neutralizing titre of saliva was less than that of gastric juice but the titre of both varied with that of the serum. Immunoelectrophoretic examination of the salivas and gastric juices indicates that both contain beta and gamma globulins and that their neutralizing titre is directly related to their protein content. Local neutralizing activity of this kind may well influence the effectivity of live (oral) vaccine and play a significant role in protection against enterovirus infection.

The Adjuvant Effect of Endotoxin on Protein Hypersensitivity and its Modifying Effect on Homologous Protein. By T. G. Kováts (*Pharmacological Institute, Medical University, Szeged, Hungary*)

The present experiments demonstrate that endotoxin may exert an adjuvant effect on cutaneous and to a lesser extent on systemic hypersensitivity in rats. Rats sensitized by subcutaneous injection of 10 μg. of crystalline ovalbumin, with and without purified typhoid endotoxin, were tested 12 days later for sensitivity to ovalbumin by intradermal injection. Those given endotoxin as well as ovalbumin were 100-fold more sensitive than those given ovalbumin alone. In contrast, the addition of endotoxin to ovalbumin only moderately enhanced the development of systemic anaphylaxis in rats. The adjuvant effect of endotoxin in this type of hypersensitivity reaction is probably attributable to its adjuvant effect on antibody formation. Apart from its adjuvant effect on foreign proteins, endotoxin can markedly modify the response to homologous protein. Thus injection of rabbits with a mixture of homologous myocardium homogenate and endotoxin induced a chronic connective tissue proliferation accompanied in some animals by Ashoff-like nodules. Similarly, 40 % of rabbits injected with homologous liver homogenate and endotoxin developed histological evidence of chronic liver injury and necrosis. Although the lesions evoked by the homologous tissue homogenate with endotoxin were chiefly localized in the organs corresponding to the source of the homogenate, other organs might also be involved. Control animals injected with tissue homogenates without endotoxin, or with endotoxin alone, did not develop significant lesions. It seems likely that the lesions described above reflect a modifying action of endotoxin on homologous protein, causing it to become 'foreign' and antigenic.

The Action of Vitamin K in Bacteria. By M. Lev (*National Institute for Research in Dairying, Reading*) and A. F. Brodie (*Harvard Medical School, Boston, Mass., U.S.A.*)

Fusiformis (*Bacteroides*) *nigrescens*, an obligate anaerobe, has previously been shown to require vitamin K. When vitamin K_1 or menadione is added to the medium, vitamin K cannot be extracted from cells grown in it. However, a compound with an absorption maximum at 260 mμ is found. This peak moves to

290 mμ at an alkaline pH. The compound satisfies the K requirement of both *F. nigrescens* and the K requiring mutant of *Haemophilus parainfluenzae*. When cell-free extracts of *F. nigrescens* are incubated with vitamin K_1, menadione, vitamin K and phthiocol, the spectra of the active forms, K_1, menadione and K_5, are altered. Phthiocol which acts as an antivitamin is unchanged after incubation. This metabolism of active forms of vitamin K suggests a anaerobic function distinct from that proposed in aerobic oxidative phosphorylation systems.

Isolation of Bacteriophage Lytic to Mycobacteria by the Soil-Enrichment Method. By J. F. COSTER (*Rijksinstituut voor de Volksgezondheid, Utrecht*) and A. G. SCHUITEMAKER (*Central Ziekenhuis, Alkmaar*)

Soil samples have been used for enrichment with mixtures of *Mycobacterium* cultures. Two groups of mycobacteria were used. The first group contained mycobacteria obtained from Professor Penso (Rome): *M. rabinowich*, *M. minetti*, *M. phlei*, *M. smegmatis*, *M. lacticola*, *M. pellegrini* and *M. battaglini*. The second group contained two strains of *M. marinum*, two of *M. platipoecilos*, four of *M. balnei* and one of *M. fortuitum*. From such enriched-soil-samples, incubated at 37° for 5 weeks, two bacteriophages were isolated by centrifuging the cultures and filtering the supernatants through Chamberland no. III filters. The phages were lytic to *M. smegmatis* and *M. lacticola*. One (CS 5) produced plaques with a sharp edge; the other (CS 6) plaques with a diffuse edge. Antisera to the newly isolated phages, and to the phages obtained from Professor Penso (lytic to *M. smegmatis* and *M. lacticola*), were raised in rabbits. With the four antisera, neutralization tests were performed by mixing bacteriophage in different dilutions with serum at a dilution 1:100, and incubating at 37° for half an hour. The phages were then titrated on *M. smegmatis* by the double-layer technique. The newly isolated bacteriophages were not neutralized by antiserum against the *smegmatis* phage or the *lacticola* phage (from Penso) but completely cross-neutralized each other. The *lacticola* and *smegmatis* phages showed no cross-neutralization at all.

A Search for the Transmission of Megacinogenic Factors in *Bacillus megaterium*. By I. B. HOLLAND and C. F. ROBERTS (*Microbiology Unit, Department of Biochemistry, University of Oxford*)

Eighteen strains of *Bacillus megaterium* producing the bacteriocin 'megacin' have been identified among 50 strains isolated from soil and 8 strains obtained from other laboratories. Those producing megacins showed considerable differences in the morphology of the inhibition zones exhibited and in their spectrum of activity against a range of sensitive organisms; in some cases megacin production was u.v.-inducible (as found previously by G. Ivánovics & L. Alföldi (1955), *Acta. microbiol. Acad. Sci., Hung.* **2**, 275). It is suggested that there must therefore be a number of different megacins. Transmission of megacinogeny from a producer strain to a streptomycin-resistant mutant of a non-producer was sought in mixed culture experiments of the type reviewed by S. M. Smith & B. A. D. Stocker ((1962), *Brit. med. Bull.* **18**, 46). There was sometimes poor recovery of the non-producers although they grew normally in separate control cultures. The producers in fact may be divided into three types: (1) non-u.v.-inducible strains which do not kill

non-producers by 14 hr. in mixed culture; (2) u.v.-inducible strains in which killing of the non-producer occurs only after it has multiplied for at least 30 min.; (3) u.v.-inducible producers which kill non-producers within 15 min. of mixing logarithmically growing cultures. Experiments in which organisms were grown in a common medium but separated by bacterial filters showed that actual contact between producer and non-producer is necessary for rapid killing. There is no evidence that megacin, at any rate acting alone, is responsible for the rapid killing described in (3) above. No transmission of factors controlling megacin production has yet been detected in long term mixed cultures using 7 group (1) producing strains. In each experiment about 600 colonies of the 'recipient' organism were tested.

Kinetics of F-Curing by Acridine Orange in *Escherichin coli*. By A. H. STOUTHAMER and P. G. DE HAAN. *Laboratory for Microbiology, State University, Utrecht.*

In 1957, Y. Hirota & T. Iijima (*Nature, Lond.* **180**, 655) reported that F^+ strains of *Escherichia coli* were converted to F^- by acridine dyes. We have studied the kinetics of this phenomenon by curing P 678 $Fgal^+$ with acridine orange (AO). The disinfection has been followed by plating on triphenyltetrazolium-chloride-galactose-streptomycin plates. On this medium P 678 F^- gives red colonies because this strain is gal^- and streptomycin resistant; P 678 $Fgal^+$ gives white colonies. At first we tested the influence of AO on cells which had just received the $Fgal^+$ factor by conjugation with a streptomycin sensitive donor strain. The multiplication of the P 678 $Fgal^+$ cells shows a lag of 20–30 min. and then the number of $Fgal^+$ cells increases at a much slower rate than the total population. In the absence of AO we found that the number of $Fgal^+$ cells increases immediately and as rapidly as the total population, indicating that AO inhibits the multiplication of F particles. We then studied the influence of AO on cells that had harboured the $Fgal^+$ factor for many generations. In these experiments the first F^- cells appeared after 1–2 generations and the probability for the segregation of F^- cells increased gradually during the incubation. Only after about 6 generations did this probability become the same as in the previous experiment. From this we infer that under normal conditions the $Fgal^+$ cells contain several copies of the $Fgal^+$ factor. Cells which have just received the $Fgal^+$ factor and which are incubated for about 30 min. in the absence of AO behave in the same way as cells that have harboured the $Fgal^+$ factor for many generations. This indicates that the number of F particles per cell increases rapidly after infection.

Tissue Culture as an Aid in the Rapid Diagnosis of Smallpox and other Virus Diseases of the Skin. By F. DEKKING (*University of Amsterdam, Netherlands*)

For the isolation of viruses of the pox group and herpes the egg membrane is mostly used. Tissue culture methods, however, may contribute to the more rapid diagnosis of these infections. In the case of herpes, the direct inoculation of vesicle fluid into tubes containing only a few, fast-growing, cells, may make a diagnosis possible within 8–18 hr. Tissue cultures cells, infected with vaccinia or variola virus, may show a specific haemadsorption of chicken red cells within 24 hr. after infection, i.e. 1–3 days before cytopathic changes become obvious. The sensitivities

of tissue culture systems and egg membranes are comparable. The egg membrane, however, is superior because it allows easy differentiation between the members of the pox group. With varicella virus in tissue culture positive haemadsorption may be observed; but this phenomenon appears later, is always associated with cytopathic changes, and will not occur in transformed cell-lines in which varicella virus will grow with difficulty if at all. The additional use of tissue culture may contribute to the rapid and reliable diagnosis of smallpox.

Scrapie. A Transmissible Disease of Sheep of Obscure Etiology. By W. S. GORDON (*Agricultural Research Council Field Station, Compton, near Newbury, Berkshire*)

Scrapie is a fatal sheep disease known in several European countries for over two centuries. More recently, imported sheep from Britain have introduced it to North America, Australia and New Zealand. Symptoms include scratching and rubbing, inco-ordination of gait and head tremor. Pathological lesions occur in the central nervous system and consist of a degeneration of nerve cells in the brain stem, a prominent astrocytosis and characteristic vacuolation of neurons. Workers at Moredun Institute (Stamp, J. T. (1962), *Vet. Rec.* **74**, no. 12, 357), at Compton (Gordon, W. S. (1959), *Proc. 63rd Annual Mtg U.S. Livestock Sanitary Ass.*, San Francisco, Dec. 1959), at the Nuffield Institute for Medical Research (Draper, G. J. & Parry, H. B. (1962), *Nature, Lond.* **195**, 670; Parry, H. B. (1962), *Heredity*, **17**, 75), and at Cambridge (Palmer, A. C. (1959), *Vet. Rev. Annot.* **5**, 1) have been investigating scrapie for many years. They have shown the disease is transmissible, has a long incubation period and that the agent is filterable, can be passed in series indefinitely through sheep ((Wilson, D. R., Anderson, R. D. & Smith, W. (1950), *J. comp. Path.* **60**, 267), goats (Pattison, I. H., Gordon, W. S. & Millson, G. C. (1959), *J. comp. Path.* **69**, 3) and mice (Chandler, R. L. (1962), *Lancet*, i, 107–8) and is remarkably resistant to heat and chemical agents. Circulating antibody to the scrapie agent has never been detected and results of experiments with sheep repeatedly reveal marked variation in susceptibility. These facts raise the possibility of genetic constitution determining susceptibility, though some believe the disease itself to be hereditary. Colour is lent to the second alternative by the pattern of naturally occurring scrapie in a family of Suffolk sheep maintained in a scrapie environment but, in families of Cheviot sheep, bred in isolation and drawn from a population in which scrapie does not occur spontaneously, it seems clear that there are heritable factors that determine susceptibility to experimental infection. The Cheviots exemplify much similar evidence being accumulated at Compton but firm conclusions regarding the natural spread of the disease await the completion of current work, which aims at establishing sheep flocks classified for their susceptibility to experimental scrapie. With animals of predictable susceptibility available, the investigation can advance on lines which hitherto have been impossible.

Observations on Experimental Scrapie in Goats. By I. H. PATTISON (*Agricultural Research Council Field Station, Compton, near Newbury, Berkshire*)

The wide variation in susceptibility (that seldom exceeds 50 %) of sheep to experimental scrapie has hampered investigation of that disease. Goats, however, have proved to be 100 % susceptible and have been used extensively during the past 8 years to study the nature of the disease and of the causal agent. The incubation period for the occurrence of clinical scrapie in goats ranges from 7 to 48 months, with a mean of 12 months. The causal agent—the true nature of which has not yet been determined—has been shown to be widely distributed in the tissues of affected animals, e.g. brain, C.S.F., sciatic nerve, pituitary gland, adrenal gland, salivary gland, spleen, pancreas, liver, muscle, but it has not yet been demonstrated in blood. The agent passes regularly through a Seitz E.K. filter and shows remarkable resistance to adverse physical and chemical treatments, e.g. it withstands boiling for several hours, and it is not destroyed by 8 % formalin. No macroscopic abnormalities have been noted in the organs of scrapie-affected animals, and histological lesions appear to be confined to the C.N.S. There is widespread neuronal degeneration, including vacuolation, in the brain stem and spinal cord, astrocytosis, and sometimes vacuolation of the 'ground substance'. During passage of scrapie through goats two distinct clinical syndromes have emerged, called 'sleepy' and 'scratching', each of which is to a large extent reproducible on inoculation into further animals. There is no obvious histopathological difference between these two clinical types. Brain inoculum from goats affected with 'sleepy' scrapie produces an encephalopathy in mice that appears to be a form of scrapie. The 'scratching' type of scrapie goat brain has not produced this mouse disease.

Transmissible Encephalopathy in Mice Inoculated with Scrapie Brain Material. By R. L. CHANDLER (*Agricultural Research Council, Compton*)

Mice inoculated with brain material from goats affected with scrapie developed a disease syndrome, characterized by signs of neurological involvement and lesions in the central nervous system. The disease was produced on each occasion when mice were inoculated with the 'drowsy' type of scrapie material, whereas normal brain material failed to produce the syndrome. There was an indication that mouse breeds differ in their susceptibility to the goat scrapie agent. The disease was serially transmissible. Brain substance from diseased mice was capable of producing typical scrapie in the goat. The clinical signs and the lesions in the central nervous system of the mouse, although differing in some respects from those in sheep and goat scrapie, were consistent in general character. The mouse disease was also consistent with scrapie in that the agent was transmissible, filterable, had a remarkable resistance to heat, could be recovered from the brain in high concentration and also from other tissues, and was infective by a variety of routes of inoculation. The disease is considered to be a form of experimental scrapie in the mouse and to resemble the natural disease so closely that it will be of use for research on scrapie. It may also be considered as an experimental disease of interest to those concerned with degenerative diseases of the nervous system in man.

Biochemical Investigations on the Nature of the Agent of 'Mouse-Scrapie'. By G. D. Hunter (*Agricultural Research Council Field Station, Compton, near Newbury, Berkshire*)

The previous abstract (Chandler, R. L. (1963), *J. gen. Microbiol.* **31**, xiv) has described the establishment of a transmissible encephalopathy ('mouse-scrapie') in mice, the source of the original inoculum being scrapie brain material from infected goats. Brain material from infected mice has been submitted to a simple procedure of cellular fractionation, and the infectivity of the various cellular fractions has been compared with that of unfractionated brain material by titration in mice. The results of this experiment show clearly that the agent is associated with particulate material, the highest levels of infectivity being found in the mitochondrial fraction and the more readily sedimentable part of the microsome fraction of the cell. Extracts of brain material (from cases of 'mouse-scrapie') treated with phenol have also proved to be infective. In titration of these extracts cases have occurred scattered rather irregularly through the dilutions. This finding contrasts with the results obtained when using the whole agent as an inoculum, and indicate that the infectivity does not depend on the survival of an intact scrapie-agent. Further experiments are in progress in an attempt to decide whether the infectivity is due to the RNA present. It does not appear likely that the infectivity resides in DNA. In the goat, ether extracts of scrapie-brain material have been shown to be infective (Pattison, I. H. & Millson, G. C. (1961), *J. comp. Path.* **71**, 101). Similar extracts from the mouse are also infective, but their infectivity is low, presumably because the agent is only moderately resistant to ether. Experiments designed to obtain a radioactive marker for the 'mouse-scrapie' agent will also be described.

Factors Influencing the Formation of Dissimilatory Nitrate Reductase by *Micrococcus denitrificans* and *Staphylococcus aureus*. By J. P. Chang and J. Lascelles (*Microbiology Unit, Department of Biochemistry, University of Oxford*)

Conditions affecting the formation of nitrate reductase, which catalyses the reduction of nitrate to nitrite, have been studied in *Micrococcus denitrificans* (Verhoeven, W., Koster, A. L. & van Nievelt, M. C. A. (1954), *Ant. van Leeuwenhoek*, **20**, 273) and a haemin-requiring mutant of *Staphylococcus aureus* (Jensen, J. & Thofern, E. (1953), *Z. Naturf.* **8***b*, 599). Nitrite formation from nitrate was assayed in cell-free extracts of *M. denitrificans* under conditions where no further reduction of nitrite occurred. Enzyme activity in anaerobic cultures was about fifty times that in aerobic cultures. Induction of the enzyme in suspensions was substrate dependent and required low aeration for maximal synthesis. High aeration repressed enzyme formation as also did chloramphenicol, *p*-fluorophenylalanine and DL-ethionine. In the mutant of *S. aureus*, the haemin requirement for either aerobic growth or anaerobic growth with nitrate as H-acceptor can be replaced by acetate, adenine, guanine and uracil (Lascelles, J. (1956), *J. gen. Microbiol.* **15**, 404). Cells transferred twice through haemin-free medium had no nitrate reductase activity and conditions necessary for induction of this enzyme were investigated. In addition to nitrate, a very low concentration of haemin (3×10^{-8} M) was required. This concentration was insufficient to activate enzyme present in adapted cells. Chlor-

amphenicol and *p*-fluorophenylalanine inhibited adaptation. Oxygen repressed adaptation even at this low concentration of haem in so it was not possible to elucidate whether oxygen requires the mediation of a functional cytochrome system to exert its effect. Cytochrome oxidase appeared to be constitutive. Oxygen did not destroy or irreversibly inhibit activity of pre-formed enzyme.

The Carbohydrate Metabolism of *Staphylococcus aureus*. By K. C. STRASTERS and K. C. WINKLER (*Laboratorium voor Microbiologie der Rijksuniversiteit, Utrecht, Netherlands*)

We have studied carbohydrate dissimilation by *Staphylococcus aureus* in Warburg experiments, by measuring enzyme activity in cell-free extracts and by experiments with radioactive substrates. Strain 3A (phage group II) has been studied in most detail. Resting cells grown in broth readily oxidize glucose, gluconate, ribose, and succinic acid. This is taken as presumptive evidence that both pentose and citric acid cycles are present. In contrast, cells grown in glucose broth do not oxidize gluconic acid, ribose or Krebs's cycle intermediates, presumably because one or more enzymes of oxidative cycles is suppressed by growth in glucose. In cell-free extracts of broth-grown cells various enzymes of the glycolytic system and the oxidative cycles were easily demonstrated. The activity of the glycolytic system in extracts of glucose-grown cells is greater than that of broth-grown cells but several enzymes of the Krebs' cycle are less active (succinic dehydrogenase) or absent (fumarase). The enzymes of the pentose cycle are about equally active in the extracts of both kinds of cells. Ribokinase and gluconokinase are, however, absent in glucose-grown cells, so explaining the inhibition of the oxidation of ribose, etc., by growth in glucose. The main products of carbohydrate oxidation are acetic acid and CO_2. Studies with radioactive substrates show that the pentose cycle is functional in broth-grown cells (64 % of the activity of glucose-1-^{14}C appearing as CO_2) and less active in glucose-grown cells (38 %). The weak link between glycolysis and the citric acid cycle implies that this cycle is used mainly for the oxidation of amino acids and not for pyruvic acid oxidation.

The Effect of Streptogramin on *Staphylococcus aureus*. By D. VAZQUEZ (*Department of Biochemistry, University of Cambridge*)

The antibiotic streptogramin was first isolated from culture filtrates of *Streptomyces graminofaciens* by J. Charney, W. P. Fisher, C. Curran, R. A. Machlowitz & A. A. Tyteli (1953, *Antib. Chemo.* 3, 1283), who showed the antibiotic is bactericidal and preferentially active against Gram-positive bacteria. This work was carried out with *Staphylococcus aureus* strain Duncan for which the minimum growth inhibitory concentration of streptogramin is 0·6 μg./ml. Streptogramin has no effect on endogenous respiration, oxidation of ethanol or glucose, or anaerobic fermentation of glucose. Cell-wall synthesis was also unaffected by streptogramin. Net synthesis of nucleic acids is stimulated in the presence of streptogramin and this stimulation is only due to an increase in rate of ribonucleic acid formation, the synthesis of deoxiribonucleic acid being unaffected. Streptogramin at the minimum growth inhibitory concentration inhibits net protein synthesis by 70 %. All these results are similar

to those obtained by Gale, E. F. & Folkes, J. P. (1953, *Biochem. J.* **53**, 493), and other workers with chloramphenicol, aureomycin, terramycin, and erythromycin. However, all these antibiotics are bacteriostatic whereas streptogramin is bactericidal. That would suggest that the primary point of action of streptogramin is not identical to that of the other antibiotics. Chloramphenicol and erythromycin completely inhibit the bactericidal action of streptogramin. The protective effect of chloramphenicol against the bactericidal action of penicillin and streptomycin has been reported by some workers and it has been suggested by T. D. Brock (1961, *Bact. Rev.* **25**, 38) that it could be due to the inhibition of bacterial growth by chloramphenicol. However that may not be the only reason for the protection of chloramphenicol against the bactericidal effect of streptogramin because aureomycin and terramycin, although bacteriostatic themselves, enhance the bactericidal effect of streptogramin. Both aureomycin and terramycin showed an additive effect with streptogramin.

Influence of Plants on Denitrification. By J. W. Woldendorp (*Laboratory of Microbiology, Wageningen, Netherlands*)

In experiments with sods derived from permanent grassland it was shown that considerable losses occurred after additions of ^{15}N-labelled nitrate. These losses were ascribed to denitrification. When the roots of the grass plants were killed by clipping the herbage several times, the losses appeared to be much smaller. These results indicate a quantitative influence of the living root system on denitrification in soil. This influence may be explained in different ways. The living roots in collaboration with the rhizosphere organisms may increase oxygen consumption of the total soil system sufficiently to produce the anaerobic conditions needed for denitrification. Apart from this, the roots may excrete products that serve as hydrogen donors in denitrification. The oxygen consumption of living permanent-grassland sods appeared to be 25 times higher than that of dead sods; 65 % of this enlarged consumption was accounted for by the roots, the remainder by rhizosphere organisms. It is concluded that the living root system promotes denitrification by lowering the quantity of dissolved oxygen in the soil solution. By using the Warburg technique, it was shown that perennial ryegrass and pea plants, grown in small baskets, filled with non-sterile artificial soil, freed from organic matter, excreted considerable amounts of hydrogen donors, as a result of which nitrous oxide and gaseous nitrogen were formed from added nitrate. The same technique was used with sterile pea plants inoculated with different species of denitrifying bacteria. In the latter case *Pseudomonas* strains exhibited the same denitrification pattern as peas grown in non-sterilized soil. *Bacillus licheniformis*, however, produced no gaseous products from nitrate but only nitrite.

Control of Carboxydismutase Formation in the Thiorhodaceae. By R. E. Hurlbert (*Microbiology Unit, Department of Biochemistry, University of Oxford*)

Recent work has suggested that the enzymes of the reductive pentose pathway of Thiorhodaceae may be adaptive (Fuller, R. C., Smillie, R. M., Sisler, E. C. & Kornberg, H. L. (1961), *J. biol. Chem.* **236**, 2140), as has been demonstrated with a number of other facultative autotrophs (Fuller, R. C. & Gibbs, M. (1959), *Plant*

Physiol. **34**, 324; Kornberg, H. L., Collins, J. F. & Bigley, D. (1960), *Biochim. biophys. Acta*, **39**, 9; Lascelles, J. (1960), *J. gen. Microbiol.* **23**, 499; Quayle, J. R. & Keech, D. B. (1960), *Biochem. J.* **75**, 515). The effect of growth conditions on the activity of ribulose 1,5-diphosphate carboxylase (carboxydismutase), a key enzyme in this pathway, has now been studied in two species of Thiorhodaceae (*Chromatium* and *Thiopedia*). Compared to cells grown autotrophically on bicarbonate and thiosulphate, carboxydismutase was repressed by growth on a variety of organic substrates. The degree of repression was never greater than 80 % and in the majority of cases was no more than 50 %. The pattern of repression did not bear any relation to the oxidation state of the organic compound tested. Carbon dioxide was not required for growth on a number of the compounds tested, nor did its addition to the growth media influence the enzyme level. Kinetic studies were used to investigate the adaptive nature of the enzyme in the *Chromatium* sp. The enzyme level of autotrophically grown cells declined immediately, and before growth occurred upon transfer to growth media containing pyruvate, and conversely increased rapidly in pyruvate-grown cells transferred to autotrophic media. The data indicate that the enzyme is destroyed during adaptation from autotrophic to heterotrophic growth. Further kinetic studies suggest that thiosulphate exerts a protective effect on the enzyme in the presence of pyruvate. The pattern of $C^{14}O_2$ incorporation in pyruvate grown cells is being investigated to determine if the carboxydismutase present is functioning.

Sheath-Forming Iron Bacteria. By E. G. MULDER (*Laboratory of Microbiology, Wageningen, Netherlands*)

Sheath-forming iron bacteria are found as flocculent masses covering submerged plants in slowly running iron- and manganese-containing waters. The brownish material consists of bacterial sheaths and iron and manganic oxides precipitated in, on, or outside the sheaths. Thirty-five strains of ensheathed bacteria belonging to the genera *Sphaerotilus* and *Leptothrix* were isolated and their morphological and physiological characteristics studied. To grow these organisms in synthetic media, vitamin B_{12} was found to be required. This was the case in media with glucose and with inorganic nitrogen or aspartic and glutamic acids as the nitrogen source. When hydrolysed casein or a mixture of L-amino acids was supplied, the requirement for vitamin B_{12} was eliminated. Methionine was found to be responsible for this effect. The thirty-five strains belong to five different types. The first one is the sewage bacterium *Sphaerotilus natans*, an organism with large cells, which responds to relatively high concentrations of organic substrates. It is unable to oxidize manganous compounds, but in running sterile iron-containing ditch water, iron oxide is deposited in and on its sheaths. Although *S. natans* under such conditions more or less resembles the iron bacterium *Leptothrix ochracea*, the two organisms were clearly different. This conclusion is in contrast to Pringsheim's statement (1949, *Biol. Rev.* **24**, 200) that they are identical. Types II and III are able to oxidize manganous compounds. They do not respond to high concentrations of organic substances. Type II resembles *L. lopholea*, type III *L. ochracea*. Type IV is an intermediate form resembling *S. natans* when it is grown with large amounts of organic substrates and type V when it is grown in the presence of manganous

compounds. Type V (*L. discophora*) has small cells, grows poorly, but has a strong manganese-oxidizing capacity. None of the isolated organisms was able to grow in a medium containing inorganic salts, manganous carbonate and vitamin B_{12}.

Control of the Methylation of Homocysteine in *Escherichia coli*. By M. A. Foster, R. J. Rowbury and D. D. Woods (*Microbiology Unit, Department of Biochemistry, University of Oxford*)

The formation of methionine from homocysteine and serine by intact organisms or ultrasonic extracts of *Escherichia coli* PA 15 (a serine or glycine auxotroph) is greatly reduced if the organisms are grown in the presence of methionine (Rowbury, R. J. & Woods, D. D. (1961), *J. gen. Microbiol.* **24**, 129). Two enzymes, in addition to serine hydroxymethylase, are necessary for the over-all reaction with cell-free extracts and an extract of heated organisms as a source of folic acid cofactor (Foster, M. A., Tejerina, G. & Woods, D. D.; to be published). Enzyme A catalyses the formation from hydroxymethyltetrahydrofolate derivatives of methyl groups which enzyme B transfers to homocysteine. The formation of both these enzymes has now been found to be reduced by growth with methionine; enzyme A, normally present in excess, is, however, repressed by concentrations of methionine which do not affect the level of enzyme B. On transfer of enzymically competent organisms to media containing methionine, the synthesis of enzyme A can be repressed independently or together with that of enzyme B according to the methionine concentration. When fully repressed organisms are transferred to methionine-free medium, both enzymes are formed rapidly before significant growth occurs. Levels of both enzymes higher than normal are found transiently during relief from repression. The formation of both enzymes requires *de novo* protein synthesis since it is blocked by chloramphenicol or by omission of glycine from the medium. Under all conditions tested, enzyme A is present in excess whereas enzyme B is present only in sufficient amount to account for the over-all synthesis of methionine. It is suggested that, although both enzymes are susceptible to repression, the methylation of homocysteine is controlled by the level of enzyme B.

Nutritional Studies on Thermophilic Bacteria. By J. E. Ford and D. J. Jayne-Williams (*National Institute for Research in Dairying, Reading*)