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Contents

(No. 1 *issued* 6 *January* 1964)

PAGE

Transduction of Swarming in *Proteus mirabilis* BY J. N. COETZEE . . 1

The Inhibition by Streptomycin of Certain Streptococcus Bacteriophages, using Host Bacteria Resistant to the Antibiotic BY T. D. BROCK, J. MOSSER and B. PEACHER 9

The Physiology and Nutrition of some Aquatic Hyphomycetes BY D. R. THORNTON 23

Effect of Environmental Conditions on the NADP-Specific Glutamic Acid Dehydrogenase in *Neurospora crassa* BY R. W. BARRATT . . . 33

An Electron-Microscope Study of Germination of Conidia of *Botrytis cinerea* BY L. E. HAWKER and R. J. HENDY 43

Size of Inoculum and Carbon Metabolism in some Aspergillus species BY J. MEYRATH and A. F. MCINTOSH 47

Size of Inoculum and Nitrogen Metabolism in *Aspergillus oryzae* BY A. F. MCINTOSH and J. MEYRATH 57

Flagellar Synthesis in *Salmonella typhimurium*: the Incorporation of Isotopically-Labelled Amino Acids into Flagellin BY D. KERRIDGE . . 63

Cultural and Serological Studies on *Haemophilus vaginalis* BY D. L. REDMOND and E. KOTCHER 77

Comparison of Cultural and Immunofluorescent Procedures in the Identification of *Haemophilus vaginalis* BY D. L. REDMOND and E. KOTCHER 89

Isolation of *N*-Acetylneuraminic Acid and 4-Oxo-norleucine from a Polysaccharide Obtained from *Citrobacter freundii* BY G. T. BARRY, F. CHEN and E. ROARK 95

A Method for the Purification of Bacterial Flagella by Ion Exchange Chromatography BY R. J. MARTINEZ 115

Analysis by Transduction of Mutations affecting Penicillinase Formation in *Staphylococcus aureus* BY R. P. NOVICK 121

The Production of Riboflavin and D-Arabitol by *Debaryomyces subglobosus* BY F. B. ANDERSON and G. HARRIS 137

Microbial and Biochemical Changes during the Moulding of Hay BY P. H. GREGORY, M. E. LACEY, G. N. FESTENSTEIN and F. A. SKINNER . . 147

Proceedings of the Nottingham Meeting of the Society i

(No. 2 *issued* 11 *February* 1964)

The 80th Birthday of Muriel Robertson 175

Some Aspects of the Protozoa and their Way of Life. The Sixth Marjory Stephenson Memorial Lecture BY M. ROBERTSON. 177

PAGE

Antibiotics from *Aspergillus amstelodami* BY W. M. DARLING, P. J. CAMPBELL and M. MCARDLE 191

The Effect of a Shift on the Frequency of Transduced *Salmonella typhimurium* Cells in the Early Period of Transduction BY J. HUBÁČEK . 205

Carbohydrate Metabolism of *Staphylococcus aureus* BY K. C. STEASTERS and K. C. WINKLER 213

Transfer of *colE*1 and *colE*2 during High-Frequency Transmission of *colI* in *Salmonella typhimurium* BY S. M. SMITH, H. OZEKI and B. A. D. STOCKER 231

Deoxyribonucleic Acid Base Composition of Acetic Acid Bacteria BY J. DE LEY and J. SCHELL 243

The Biosynthesis by Pneumococcus of a Non-Reducing Disaccharide from Uridine Diphosphoglucose BY E. E. B. SMITH and G. T. MILLS . . 255

The Classification of 'Bacterium salmonicida' BY I. W. SMITH . . . 263

The Serological Identification of Streptomycetes by Agar Gel Diffusion Techniques BY T. CROSS and D. F. SPOONER 275

Radiosensitivity in *Escherichia coli* BY J. GREENBERG and P. WOODY-KARRER 283

Sexual Polarity in *Streptomyces coelicolor* BY G. SERMONTI and S. CASCIANO 293

Investigations about the Formation of Incomplete Forms of Fowl Plague Virus BY R. ROTT and C. SCHOLTISSEK 303

Sodium Azide Resistance in *Proteus hauseri* BY J. N. COETZEE, H. C. DE KLERK and I. J. MARÉ 313

L forms of *Staphylococcus aureus* BY R. E. O. WILLIAMS 325

Dry Liberation of Fungus Spores by Raindrops BY J. M. HIRST and O. J. STEDMAN 335

Observations on *Sphaleromantis tetragona* BY K. HARRIS 345

(No. 3 *issued* 18 *March* 1964)

Taxonomy of the Species *Pseudomonas odorans* BY I. MÁLEK, M. RADOCHOVÁ and O. LYSENKO 349

The Flocculation of Suspended Matter by *Paramecium caudatum* BY C. R. CURDS 357

Isolation of Auxotrophs of *Penicillium chrysogenum* and their Penicillin Yields BY K. D. MACDONALD, J. M. HUTCHINSON and W. A. GILLETT . 365

Heterokaryon Studies and the Genetic Control of Penicillin and Chrysogenin Production in *Penicillium chrysogenum* BY K. D. MACDONALD, J. M. HUTCHINSON and W. A. GILLETT 375

PAGE

Formation and Segregation of Heterozygous Diploids between a Wild-Type Strain and Derivatives of High Penicillin Yield in *Penicillium chrysogenum* BY K. D. MACDONALD, J. M. HUTCHINSON and W. A. GILLETT . . 385

The Chemical Composition of Lambda in *Paramecium aurelia*, Stock 299 BY W. J. VAN WAGTENDONK and R. B. TANGUAY 395

The Cultivation of Some Rumen Oligotrich Protozoa BY R. T. J. CLARKE . 401

Sequential Ascus Collection in *Neurospora crassa* BY W. N. STRICKLAND and D. THORPE 409

Sensitization of Bacterial Spores to Lysozyme and to Hydrogen Peroxide with Agents which Rupture Disulphide Bonds BY G. W. GOULD and A. D. HITCHINS 413

An Electron Microscope Study of Root-Hair Infection by Rhizobium BY K. SAHLMAN and G. FÅHRAEUS 425

Nuclear Morphogenesis During the Developmental Cycles of Some Members of the Genus *Nocardia* BY J. N. ADAMS 429

Studies on the Specificity of Interferon BY G. E. GIFFORD 437

Ribulose Diphosphate Carboxylase in Thiorhodaceae BY R. E. HURLBERT and J. LASCELLES 445

Fatty Acid Requirements of *Mycoplasma laidlawii* BY S. RAZIN and S. ROTTEM 459

Osmotic Lysis of Mycoplasma BY S. RAZIN 471

Chemical Composition of Mycoplasma Cells and Membranes BY S. RAZIN, M. ARGAMAN and J. AVIGAN 477

The Effect of Impure Tetanus Toxin on the Frequency of Miniature End-Plate Potentials BY G. A. FEIGEN, N. S. PETERSON, W. W. HOFMANN, G. H. GENTHER and W. E. VAN HEYNINGEN 489

Books Received 497

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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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.

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Transduction of Swarming in *Proteus mirabilis*

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(*Received* 27 *December* 1962)

SUMMARY

A phage able to transduce a streptomycin resistance marker in *Proteus mirabilis* can also separately transduce the swarming characteristic between variants of two strains of *P. mirabilis*. Motile non-swarming variants were made to swarm on agar by incorporation of a swarming gene via a phage lysate of a swarming strain. The production of swarms by motile non-swarming variants when treated with phage lysates of other phenotypically similar strains indicated that at least three non-homologous factors control swarming. An O variant could also act as a donor of the swarming gene. This is because the O strain possesses an intact swarming centre which is masked by the absence of active flagella. This O variant was transduced to swarming by phage lysates of motile non-swarming strains or of a swarming strain. The gene transduced here was concerned with the presence of flagella. Factors controlling two morphological varieties of swarming were separately transduced to suitable recipients and a locus able to modify wild-type swarms was identified.

INTRODUCTION

Many organisms may swarm under suitable conditions. Thus Salmonella (Stocker, Zinder & Lederberg, 1953), *Escherichia coli* (Furness, 1958) and members of the genus *Bacillus* (Brown, Cherry, Moody & Gordon, 1955) swarm on semi-solid agar. *Proteus morganii* swarms on 1 % agar at 20–28° (Rauss, 1936) and members of the genus *Clostridium* may swarm, particularly on moist media (Fildes, 1925). The A and C phases (Belyavin, 1951) of *P. hauseri* are able to swarm vigorously on well-dried nutrient agar.

Many explanations for the swarming of *Proteus hauseri* have been given (Lominski & Lendrum, 1947), but the phenomenon is now generally attributed to negative chemotaxis exerted by toxins which form during the growth of the culture (Lominski & Lendrum, 1947; Hughes, 1957). These toxins have not been identified. As regards the mechanism of swarming it is agreed (*Topley & Wilson*, 1955) that non-motile organisms do not swarm; active flagella are a prerequisite. Coetzee & Sacks (1960*a*) reported that certain temperate Proteus phages could transduce a streptomycin resistance marker. Swarming and non-swarming variants of some of the strains used were available (Coetzee, 1959; Coetzee & Sacks, 1960*b*), and it was decided to apply transduction techniques to the study of the phenomenon of swarming in *P. mirabilis*.

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METHODS

Media. The composition of the broth and the MacConkey-type agar employed have been described (Coetzee & Sacks, 1960*b*). In addition, a nutrient agar made and used routinely by the Department was used. In some experiments to detect abortive transductants the agar concentration in media was reduced to 0·6 % (w/v). Plates were well dried before use. The incubation temperature was 37°.

Organisms. Variants of *Proteus mirabilis* strains 13 and 193 were used (Coetzee, 1959; Coetzee & Sacks, 1960*a*, *b*). These were 13-Y, 13-W_1, 13-W_7, 13-Z, 13-O_1, 13-Y *str-r*, 193-Y, 193-W_4, 193-W_{15}. After 12 hr. incubation organism 13-Y has swarmed vigorously across nutrient agar or MacConkey plates in two or three bounds (Pl. 1, fig. 1). Variant 13-Z swarms as vigorously but in one continuous sheet of growth (Pl. 1, fig. 2). The Y and Z variants correspond respectively to the A and C phases of Belyavin (1951). Organism 193-Y swarms in compact rings and only covers half the area of a plate in the same period of time (Pl. 1, fig. 3). It hardly swarms at all on MacConkey agar. Agglutinin absorption tests (Coetzee, unpublished) showed that the H antigens of organisms 13 and 193 were identical while the O antigens differed. This difference could possibly be reflected in the fact that strain 13 is lysed by Proteus phages 63, 78 (Coetzee, 1958), while strain 193 lacks these phage receptors. The W forms of these two strains are motile and non-swarming and correspond closely to the B phase described by Belyavin (1951). They agglutinate to titre with 13-Y or 193-Y H sera. O variants—i.e. non-motile strains which do not agglutinate with H sera (Friewer & Leifson, 1952) are rare in these strains and only one (13-O_1) has been isolated. The W variants mutate to corresponding Y forms at low rates (Coetzee & Sacks, 1960*b*) and the O variant does the same (Coetzee, unpublished). Strains were stored on nutrient agar slopes at 4°.

Phage. Phage 34/13 (Coetzee & Sacks, 1960*a*) was used. Phage lysates were prepared, sterilized and titrated according to methods described in the same paper. Grown on organism 13 it has a relative efficiency of plating of unity on strain 193.

Transduction techniques. The recipient organism was washed off a stock agar slope with broth and a suspension containing *c.* 1×10^9 organisms/ml. made. This was done by visual comparison with a standard suspension. One ml. was then centrifuged, the supernatant discarded and the deposit mixed with 2 ml. of phage lysate of the donor strain (plaque-forming titre *c.* 7×10^9/ml.). The mixture was incubated for 45 min. Loopfuls of the contents were then streaked on both types of solid media and the plates incubated for 15 hr. In some experiments the mixtures were diluted with broth before streaking, while in others the tubes were centrifuged and the deposits streaked out as before. Controls included a lysate sterility control and a recipient control in the form of a lysate prepared on the recipient organism and mixed with that organism as above. As phage lysates of donor organisms might perhaps contain swarm-inducing toxins for the recipient (Hughes, 1957) an additional control was used. This consisted of the supernatant obtained by centrifuging a 4 hr. growth of the donor organism in soft top-layer agar used in lysate preparation, sterilized with chloroform, as were phage lysates, and mixed with the recipient organism as described above. Lysates which caused swarming of recipient organisms were treated with deoxyribonuclease and anti-phage serum as previously described (Coetzee & Sacks, 1960*a*). Streptomycin resistance was transduced as described

in the same paper. Methods used to measure phage adsorption and to detect lysogeny in transductants were also described in the paper. The character of swarms which appeared in these experiments was investigated by subculturing them on to fresh agar plates.

Serological techniques. These were as described for Proteus by Kauffmann (1951).

RESULTS

Transduction studies. Phage controls were always sterile. The two recipient controls usually showed no swarms after 15 hr. incubation (Pl. 2, fig. 4) but often produced a couple of swarms after a further 12–24 hr. No differences were noted between the two types of recipient controls. A summary of experiments performed and the nature of the swarms evoked from recipient organisms by phage lysates is presented in Table 1. Numerous swarms were evoked from variants 13-O_1, 13-W_1, and

Table 1. *Summary of results of transduction experiments with phage* 34/13

Donor	Recipient	Type of swarm evoked
13-Y	13-O_1, 13-W_1, 13-W_7, 193-W_4, 193-W_{15}	13-Y
13-Y*str-r*	13-O_1, 13-W_1, 13-W_7, 193-W_4, 193-W_{15}	
13-O_1	13-W_1, 13-W_7, 193-W_4, 193-W_{15}	
13-W_1	13-O_1, 13-W_7, 193-W_4, 193-W_{15}	
13-W_7	13-O_1, 13-W_1, 193-W_4, 193-W_{15}	
13-Z	13-O_1, 13-W_1, 13-W_7, 193-W_4, 193-W_{15}	
13-Z	193-Y	Z, 13-Y

13-Y swarming = discontinuous broad zone. 193-Y swarming = discontinuous compact. Z swarming = continuous.

13-W_7 by treatment with phage lysates of 13-Y or 13-Y *str-r* (Pl. 2, figs. 5, 6). No quantitative work was done, but the number of swarms produced by the above systems appeared to be of a similar order—about 20/streak. All the swarms had the 13-Y (broad zone) mode of spread. The swarm-inducing abilities of these lysates and all other lysates used in this investigation were not affected by prior treatment with deoxyribonuclease but were completely abolished by an exposure to homologous phage antiserum which reduced the plaque-forming titre below 10^6/ml. It is concluded that the phenomena presented are examples of phage-mediated transduction. These swarms and all other transductant swarms investigated were lysogenic for strain 13 and did not adsorb phage 34/13. This is attributed to lysogenic conversion (Coetzee, 1961). Phage lysates of 13-Y *str-r* were also capable of transducing resistance to 1000μg. streptomycin/ml. in these variants (Coetzee & Sacks, 1960*a*). These two markers are apparently not very closely linked; for no joint transductants were observed amongst 200 swarms and 300 streptomycin-resistant colonies examined. Swarms of the 13-Y (broad zone) variety were evoked from variants 13-W_1, 13-W_7 by a phage lysate of 13-O_1. It would appear that 13-O_1 cannot swarm because it has lost a gene determining the presence of flagella. It retains the gene for swarming and this gene can be transduced from it to variants 13-W_1 and 13-W_7, which are motile but do not swarm. Numerous swarms were also evoked when 13-O_1 was treated with phage lysates of 13-W_1 or 13-W_7. It is probable

that in the first series as well as in the latter experiments a gene concerned with flagellation was transmitted from 13-Y, 13-W_1, 13-W_7 to 13-O_1. Swarms were evoked by phage lysates of 13-Y, 13-O_1, 13-W_1, 13-W_7 from variants 193-W_4 and 193-W_{15}. The swarms were all of the 13-Y (broad zone) type. Recipient controls showed a few swarms after 24 hr. incubation but these were all of the 193-Y (compact) type. In years of work with strain 193 it has never swarmed in any other way than the tight-ring form previously described (Pl. 1. fig. 3). The complexity of the swarming locus was further demonstrated by the finding that swarms of the 13-Y (broad zone) variety were elicited from variant 13-W_1 by treatment with phage lysate 13-W_7 and *vice versa*. The reason why all the swarms evoked from 193-W recipients by phage lysates of strain 13 and its variants should be of the 13-Y (broad zone) and not the 193-Y (compact) variety may be due to the proximity of the 193 swarming-type locus and the locus responsible for loss of swarming in the two recipients, so that both loci are replaced by their alleles present on the transducing fragment. No more W variants of strain 193 were available to test this hypothesis and it was not possible (see Discussion) to test the interaction of variants 193-W_4 and 193-W_{15}. However, the relationship between the motile non-swarming variants means that the swarming locus in these strains is composed of at least three sites. Allogenic transformations in pneumococci are well known (Jackson, 1962) and transductions of a similar nature were first described amongst a group of galactose-non-fermenting mutants in *Salmonella typhimurium* (Zinder & Lederberg, 1952). Organisms 13-O_1, 13-W_1, 13-W_7, 193-W_4, 193-W_{15} were treated with phage lysates of organism 13-Z. Numerous swarms were produced but they were all of the 13-Y (broad zone) type. In case the transduction rate of the Z type of swarming was very low the transduction mixtures were centrifuged and the deposits streaked. Again only 13-Y type swarms were detected. The following hypothesis may explain these results. Swarming of *Proteus hauseri* is normally (Coetzee & Sacks, 1960*b*) of the discontinuous 13-Y (broad zone) or 193-Y (compact) type. This swarming is controlled by a swarming locus and the 13-Y and 193-Y types are controlled by alleles Y^+_{13} and Y^+_{193} (Clowes, 1960). The Z-type continuous swarming is produced when a modifier gene (the Z gene) mutates in a Y^+ genome. This Z locus is not closely linked to the swarmer locus Y or to the locus (or loci) controlling the presence of flagella. When a 13-Z lysate is applied to 13-O_1 transductants acquiring the flagellar gene are selected and the newly flagellated organism then swarms because it possesses a latent swarming locus. When the 13-Z lysate is applied to any of the W variants transductants acquiring the swarming gene are selected, and the resultant swarms therefore manifest the Y character of the donor. Independent incorporation of the Z^+ factor remains undetected because the W recipients have defective swarming loci. This hypothesis was tested by making use of the very restricted swarming of 193-Y on MacConkey agar. A transduction experiment was done with 193-Y as recipient and a donor lysate from 13-Z (Table 1). Controls on MacConkey plates showed the restricted 193-Y swarming. The test plates had numerous large swarms after 12 hr. incubation. The periphery of 50 discrete swarms were subcultured on to nutrient agar and incubated overnight. Twenty-nine of the swarms were of the 13-Y (broad zone) type and 21 had the 13-Z (continuous sheet) character. In this experiment both the flagellation and swarming loci of the recipient organism were intact. If the recipient incorporated the exogenous swarming gene then 13-Y type swarms

resulted; when the Z^+ locus of the donor was incorporated, the swarming of 193-Y was modified to the Z pattern.

No trails of colonies (Stocker *et al.* 1953; Stocker, 1956) at a distance from the original streaks were detected even with the use of semi-solid media in the transduction of motility to variant 13-O_1. Trails could be expected if abortive transductions to swarming occurred. Because the swarms may have obscured such trails and because abortive transductions are almost always more numerous than the complete form (Ozeki, 1956) transduction mixtures were diluted before streaking. This had the effect of reducing the number of swarms/streak, but no trails of colonies were seen.

Serology. Organisms 193-W_4 and 193-W_{15} transduced to the 13-Y type of swarming by respectively phage lysates 13-Y and 13-O_1, and organism 13-O_1 transduced to swarming by a lysate from 13-Y were examined serologically. O and H sera were prepared and agglutinin absorption tests done to detect antigenic differences between the transductants or between the latter and their parent organisms, 193-Y and 13-Y. All the H antigens proved identical. The O antigens of the 13-Y type transductants of 193-W_4 and 193-W_{15} were identical but the O sera against these strains contained antibodies which were not absorbed by strains 193 or 13. Similarly, an O serum against the swarming transductant derived from 13-O_1 possessed antibodies which were not absorbed by strains 193 or 13. The differences between the transductants and their parent organisms were thought to result from lysogenic conversion (Coetzee, 1961).

DISCUSSION

Stocker *et al.* (1953) have shown that a number of distinct genes control the presence, antigenic structure and functions of flagella in Salmonella. Through lack of non-motile flagellated (paralysed) variants (Friewer & Leifson, 1952) it was not possible to identify separate loci controlling the presence of flagella and their motility-conferring properties in this investigation. Having only one O variant also made it impossible to examine the possible complexity of the flagellation locus. A nice distinction was however made between the latter locus (or loci) and the swarming locus, which was found to consist of at least three sites. It was not possible to determine whether the swarming and flagella loci were linked. The Y (ring swarming) variant is the wild type of *Proteus hauseri* (Coetzee & Sacks, 1960*b*), and this type of swarming is controlled by the swarming locus. Because phage lysates of 13-Z (continuous swarming) transduce either the 13-Y or the 13-Z type swarming to suitable recipients, the 13-Z phenotype is thought to result from the action of a modifier gene (Z^+) on the swarming locus. These two factors are not linked closely enough to be transduced together. Bryan (1961) described a modifier gene in Pneumococci whose only detectable expression was the enhancement of activity of specific streptomycin-resistance mutations.

No experiments are reported with the use of phage lysates of variants of organism 193, because phage 34/13 grown on 193 has an e.o.p. of 10^{-3} on organism 13. The plaques are clear. Clear plaques are also formed on all variants of 193 and no transductions have been observed. This change appears to result from a phenotypic modification of the phage (Luria, 1953) but has not been extensively studied. Thorne (1962) described a *Bacillus subtilis* transducing system in which the transducing phage could not be propagated on the recipient organism.

No evidence for the occurrence of abortive transductions of swarming was encountered. This is not an exceptional finding as this type of transduction has only been reported in Salmonella (see Hartman, Hartman & Šerman, 1960) and *Escherichia coli* systems (Gross & Englesberg, 1959) and not in *Pseudomonas aeruginosa* (Holloway & Monk, 1959), *Staphylococcus aureus* (Edgar & Stocker, 1961) or *Bacillus subtilis* (Thorne, 1962) transductions.

Because the flagellar antigens of organisms 193 and 13 are identical it has not been possible to analyse the genes which control H antigenic specificity. The system employing phage 12/57 and *Proteus mirabilis* strains 57 and 113 (Coetzee & Sacks, 1960*a*) would possibly have been more rewarding as these strains differed in somatic and flagellar antigens (Coetzee, unpublished) but strain 113 has been lost. Initially phage 12/57 was used. It too is capable of transducing the swarming character between Y and W variants of strain 57 but, as no other variants of this strain could be selected, the present system was chosen. Indeed the paucity of variants and the very restricted host-ranges of the transducing phages are serious obstacles in the transduction studies with *P. hauseri*.

This work has shown that phage 34/13 can independently transduce a streptomycin-resistance marker, a flagellation marker, the swarming-type and Z markers. It may now be classed as a phage capable of general transduction.

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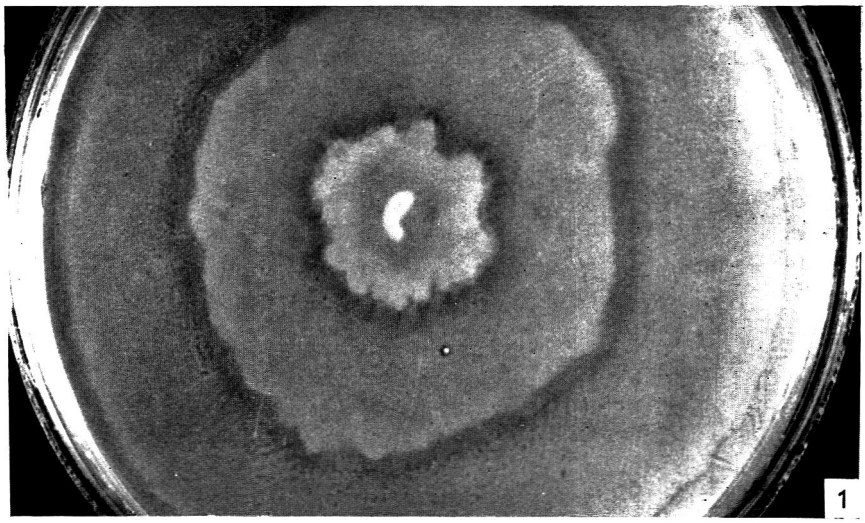

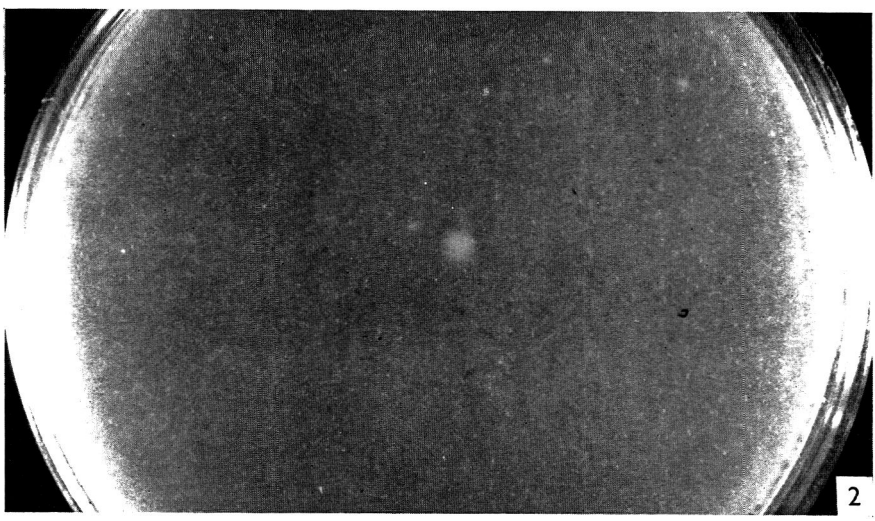

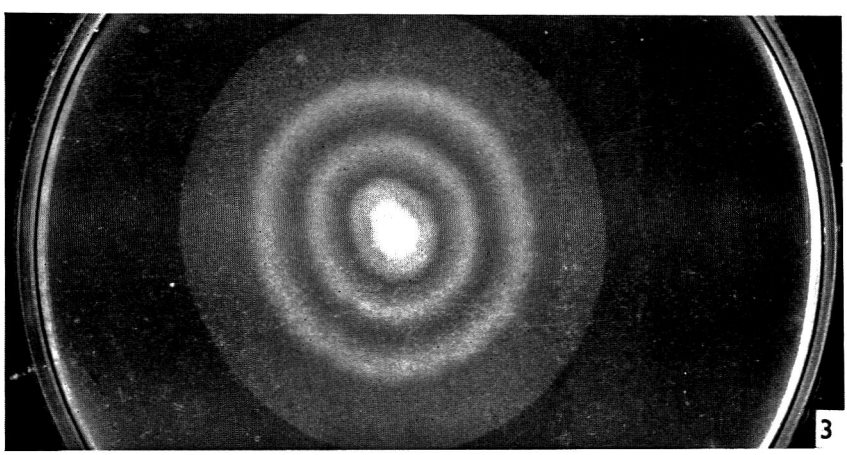

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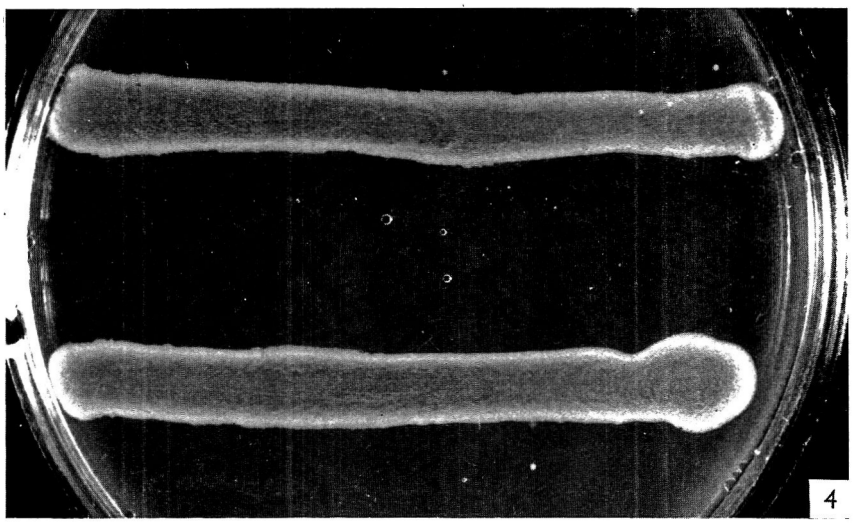
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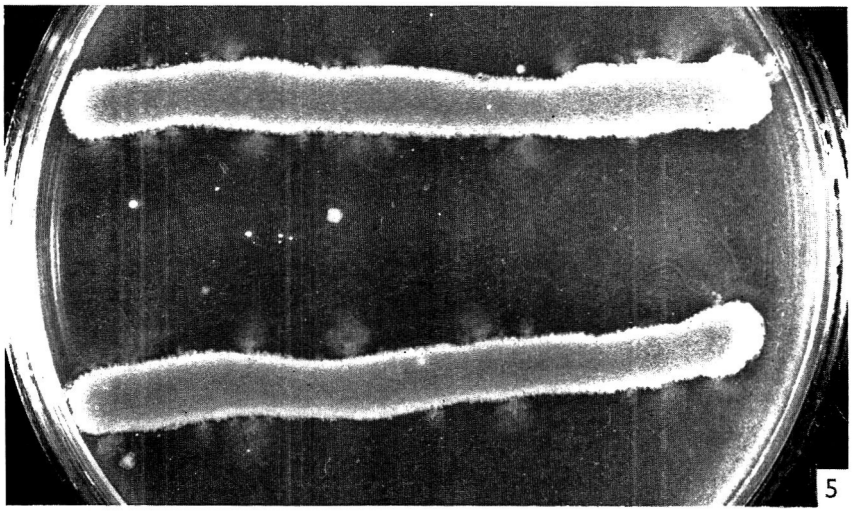
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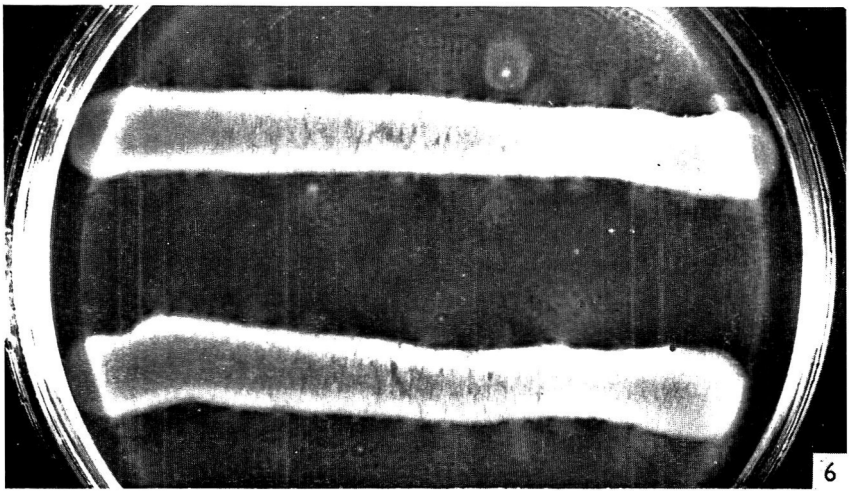
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EXPLANATION OF PLATES

(All figures life size.)

PLATE 1

Fig. 1. Organism 13-Y inoculated at centre of nutrient agar plate and incubated for 15 hr. at 37°.

Fig. 2. Organism 13-Z inoculated at centre of nutrient agar plate and incubated for 15 hr. at 37°.

Fig. 3. Organism 193-Y inoculated at centre of nutrient agar plate and incubated for 15 hr. at 37°.

PLATE 2

Fig. 4. A 13-W_1 recipient control on nutrient agar. No swarms present after 15 hr. at 37°.

Fig. 5. Organism 13-O_1 treated with a 13-Y phage lysate. Many small discrete swarms and a few large swarms are present on MacConkey agar after 15 hr. at 37°.

Fig. 6. Organism 13-W_7 treated with a phage lysate of 13-Y. Swarms have merged but their points of origin can be seen. Nutrient agar after 12 hr. at 37°.

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The Inhibition by Streptomycin of Certain Streptococcus Bacteriophages, using Host Bacteria Resistant to the Antibiotic

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SUMMARY

Streptomycin is a specific antiviral agent for a variety of bacteriophages active against *Streptococcus faecium*, *S. faecalis*, *S. liquefaciens* and *S. zymogenes* since it inhibits bacteriophage growth even when the host is resistant to the antibiotic. An analysis of the mode of action of the antibiotic in this system reveals that it probably has two effects. If the antibiotic is present before adsorption, it inhibits injection of the phage DNA. This effect is readily reversible. If the antibiotic is added after adsorption, it appears that injection is not inhibited, but that the phage genome is inactivated. The antibiotic has no effect on replication of the phage once the genome has become established in the host cell. This is consistent with the hypothesis that the phage DNA exists transiently at a streptomycin-accessible site, and then moves to a site of replication which is inaccessible to the antibiotic. Streptomycin-resistant phages probably have a different injection mechanism from streptomycin-sensitive phages.

The senior author has reported similar findings for an RNA bacteriophage of *Escherichia coli*. The implications of this work for virus chemotherapy and for analysing the mode of penetration into the cell of virus nucleic acid are discussed.

INTRODUCTION

In most studies on the effect of antibiotics on bacteriophage growth, the host cells used have been sensitive to the inhibitory agent, so that a specific antiviral effect has not been achieved. We would like to report on a specific antiviral action of streptomycin, under special conditions. This action is revealed by using host bacteria that are completely resistant to the antibiotic.

The initial observation that led to the present work arose when attempts were made to assay certain streptococcus bacteriophages on streptomycin agar, using a resistant host. No plaques were obtained. The specificity of the effect was shown by the fact that certain other bacteriophages attacking the same host were almost completely unaffected. Because of the detailed knowledge of the chemistry and biology of the bacteriophages of *Escherichia coli*, tests were made of the effect of streptomycin on replication of this group. All of the DNA viruses tested were resistant, but an RNA virus was sensitive (Brock, 1962). The data from a detailed analysis with this system were consistent with the hypothesis that the genome of the RNA phage existed transiently after adsorption at a site accessible to streptomycin, and then moved to a streptomycin-inaccessible site.

The similarity of the present findings to those with the *Escherichia coli* RNA virus will be stressed, although the streptomycin-sensitive *Streptococcus faecium* viruses appear to contain DNA instead of RNA.

MATERIALS

Bacteria

In most of the work the host bacterium was *Streptococcus faecalis* R (ATCC 8043), although this strain better fits the characteristics used to delineate *S. faecium* (Barnes, 1956). The isolate used in the present work was obtained from the Indiana University culture collection and differs in bacteriophage sensitivity from another isolate of the same culture obtained from the Sloan-Kettering Institute, labelled by them SF/O. Specifically, the I.U. isolate is sensitive to the whole range of bacteriophages discussed here, while the S.K.I. isolate is resistant to P3 and sensitive to the other viruses. The I.U. culture (designated here as x13) has been used exclusively in this work. This organism is inhibited by 250 μg. streptomycin/ml. but not by 125 μg./ml. A mutant of x13, designated x13s, resistant to 1000 μg. antibiotic/ml., was isolated by plating a large population of cells on streptomycin-agar plates and picking a colony. Other bacteria used are listed in Table 1. Streptomycin-resistant mutants for all these strains were isolated.

Table 1. *Bacteria and bacteriophages*

Bacteria	Code no.	Source
Streptococcus zymogenes	x14	I.U. Culture collection
S. zymogenes	x73	Dr C. Rogers, strain 1
S. zymogenes	x74	Dr C. Rogers, strain 2
S. liquefaciens	x33	Mr E. Buecher isolate
S. faecium	x13	I.U. Culture collection, *S. faecalis* R

Phage	Source	Host	Streptomycin sensitivity
P1	H. D. Slade	x14	Sensitive
P3	A. Kopecky Vidaver, from sewage	x13	Resistant
P4	Indiana sewage	x33	Sensitive
P5	Indiana sewage	x33	Sensitive
P6	Indiana sewage	x13	Sensitive
P7	Indiana sewage	x13	Resistant
P8	Indiana sewage	x13	Sensitive
P9	Indiana sewage	x13	Sensitive
P10	Indiana sewage	x13	Sensitive
P11	Indiana sewage	x13	Sensitive
P12	Indiana sewage	x33	Sensitive
P13	Indiana sewage	x13	Sensitive
P15	Dr Charles Rogers, phage 1	x73	Sensitive

Bacteriophage

The sources of the bacteriophages are listed in Table 1. P3, P6, P7, P8, P9, P10, P11 and P13 all attack strain x13. Bacteriophages P3 and P7 are similar in plaque size and plaque morphology, are resistant to streptomycin, and differ distinctly from the others. All of the other phages active against x13 are sensitive to streptomycin and are serologically related. Each bacteriophage can be distinguished

from all the others by its differential action on a series of phage-resistant mutants. All of the detailed work reported below has been done with P9.

Media and bacteriophage titrations

Todd-Hewitt broth and agar (Difco Laboratories, Detroit, Michigan) have been used exclusively. For bacteriophage titrations, bottom agar was 30 ml. of Todd-Hewitt broth with 1·5 % agar; top agar was 2 ml. of Todd-Hewitt broth with 0·75 % agar. All bacteriophage dilutions were made in Todd-Hewitt broth and 0·1 ml. of the final dilution added to the soft agar tube before pouring. The phage-sensitive culture (strain x13s) was grown to an O.D. of 0·500 (Bausch and Lomb Colorimeter, 640 mμ, 16 mm. tube), and kept cold until needed. 0·1 ml. was added to each soft agar tube before pouring. At an O.D. of 0·500, strain x13s had around $3{\cdot}5 \times 10^8$ chains/ml. and 6×10^8 cocci/ml. When streptomycin was to be used, it was usually added at a concentration of 1000 μg. streptomycin sulphate/ml. (Nutritional Biochemicals Co.); the appropriate amount of powder was added in dry form directly to the broth or melted agar. No attempt was made to sterilize the antibiotic and at this concentration contamination did not occur. All incubations were at 37°.

One-step growth curves

Strains x13s was grown to an O.D. of 0·500 (6×10^8 cocci/ml.) and kept cool until needed. The appropriate amount of suspension was mixed with the appropriate amount of P9 lysate in a 10 ml. total volume and incubated at 37°. After adsorption the suspension was filtered on a membrane filter (Millipore Type HA), washed with 20 ml. broth, and then suspended in 10 ml. broth. The suspension from the filter was then diluted and this dilution incubated for the growth experiments. Samples of 0·05 ml. were removed and diluted in Todd-Hewitt broth for phage assays. Duplicate samples of 0·1 ml. were treated with 0·05 ml. chloroform for 10 min. at 37° and then diluted and assayed, to give an estimate of intracellular mature phage.

Analyses

RNA and DNA analyses of phage P9 were performed on hot perchloric acid extracts of purified phage suspensions using the methods of Chargaff & Davidson (1955) and Burton (1956) respectively, using yeast RNA and thymus DNA as standards.

Antiserum

Antiserum against P9 was prepared by repeatedly injecting rabbits subcutaneously or in the foot pad with 0·5–2 ml. volumes of a mixture of equal parts Freund's adjuvant (Colorado Serum Co.) and P9 suspension that assayed 2×10^{11}/ml. The 'K' value of Adams (1959) was used to estimate the neutralizing power of the antiserums.

Binding of radioactive streptomycin

Mono-tritiostreptomycin was a gift of Dr C. Hurwitz. It was used at a specific activity of 1·44 mc./mg. and an antibiotic concentration of 1 mg./ml. in Todd-Hewitt broth. Binding was measured by incubating x13 or x13s cocci at 2×10^8 cocci/ml. with the radioactive antibiotic for various periods of time and then filtering 0·5 ml. samples on membrane filters (Millipore Type HA), washing with 2 ml.

broth, drying, and counting in a Nuclear-Chicago Co. windowless gas flow counter. Control experiments had shown that there was no self-adsorption under these conditions.

Electron microscopy

Partially purified suspensions of P9 were sprayed or dropped on collodion films, shadowed with palladium at a 3:1 angle, and examined in an RCA EMU-3C electron microscope. The phage suspensions were sometimes mixed with equal amounts of 2% potassium phosphotungstate containing 2% sucrose and sprayed on carbon grids. Other phages were observed without purification by the method of Dawson, Smillie & Norris (1962).

RESULTS

Preliminary observations on streptomycin-sensitivity of the bacteriophages

When lysates of the various bacteriophages which attack strain x13s were assayed on plain and streptomycin agar, the results in Table 2 were obtained. It can be seen that P3 and P7 were almost completely resistant, whereas P6, P8, P9, P10, P11 and P13 formed no plaques on streptomycin plates even when 0·1 ml. of undiluted lysate was plated. These results show that the action of streptomycin depends on the phage strain and not on the host bacteria, indicating that streptomycin is having a specific antiviral effect.

Table 2. *Effect of streptomycin on plaque formation of several* Streptococcus faecium *bacteriophages*

Phage strain	Plain agar		Streptomycin agar
	x13 indicator	x13s indicator	x13s indicator
	Plaque count/ml. crude lysate		
P3	$1{\cdot}8 \times 10^{10}$	$2{\cdot}3 \times 10^{10}$	$7{\cdot}8 \times 10^{9}$
P6	$4{\cdot}7 \times 10^{8}$	$2{\cdot}8 \times 10^{6}$	$< 10^{1}$
P7	$8{\cdot}6 \times 10^{9}$	$1{\cdot}2 \times 10^{10}$	$5{\cdot}5 \times 10^{9}$
P8	$2{\cdot}8 \times 10^{8}$	$2{\cdot}0 \times 10^{8}$	$< 10^{1}$
P9	$3{\cdot}7 \times 10^{9}$	$4{\cdot}2 \times 10^{9}$	$< 10^{1}$
P10	$2{\cdot}0 \times 10^{9}$	$1{\cdot}8 \times 10^{8}$	$< 10^{1}$
P11	$3{\cdot}5 \times 10^{7}$	$1{\cdot}0 \times 10^{7}$	$< 10^{1}$
P13	—	$1{\cdot}5 \times 10^{7}$	$< 10^{1}$

Phages assayed by the soft-agar method on agar with or without 1000 μg. streptomycin sulphate/ml.

Tests with bacteriophages which attack other hosts indicated that phages P1, P4, P5, P12 and P15 were also sensitive to streptomycin. However, several temperate phages which attack x33 are resistant to the antibiotic. These results further emphasize that streptomycin-sensitivity depends on the phage rather than the host cell.

Characteristics of the streptomycin-sensitive bacteriophages of strain x13

Each of the streptomycin-sensitive phages which attack x13 was an independent isolate from a different sewage sample. By isolating a series of phage-resistant x13 mutants, it could be shown that each of the phages could be distinguished by its differential action on this series of mutants. The streptomycin-sensitive phages were all similar in plaque size, plaque morphology, and rate of growth, whereas the streptomycin-resistant phages, P3 and P7, differed markedly from the others in these characteristics. All six streptomycin-sensitive phages were neutralized by P9 antiserum, whereas the streptomycin-resistant phages were not neutralized. The streptomycin-sensitive phages which attack strains other than x13 were not neutralized by P9 antiserum. Thus streptomycin-sensitivity is not confined to one serological group of phages, although all of the streptomycin-sensitive phages of x13 were serologically related.

Characteristics of the streptomycin-resistant host

In all the work to follow, x13s was used with phage P9. The complete resistance to streptomycin of the x13s host should be emphasized, since it is crucial to the hypothesis that the antibiotic is having a specific antiviral effect. The mutant formed equal numbers of colonies of equal size on both plain agar plates and on agar plates containing 1000 μg. streptomycin/ml. When 1000 μg. antibiotic/ml. was added to an exponentially growing culture, the growth rate was unchanged. The sensitive strain bound 2×10^4 molecules/coccus after 2 hr. incubation in 1000 μg. antibiotic/ml., while the resistant mutant bound about 400 molecules/coccus (this is at the limit of sensitivity of the method). The growth rate in streptomycin-broth of x13s was the same as that of x13. The resistant mutant remained sensitive to the whole range of bacteriophages attacking x13 and the efficiency of plating on both strains was about the same. Strain x13s has remained unchanged in its resistance after cultivation in the absence of the antibiotic for two years.

Characterization of phage P9

Because it had been shown in *Escherichia coli* that only an RNA bacteriophage was streptomycin-sensitive (Brock, 1962), it was of interest to determine the nucleic acid content and morphology of P9.

Four litres of P9 lysate of a titre of $2{\cdot}5 \times 10^{10}$ /ml. were prepared. After cooling, dry ammonium sulphate was added to 90 % saturation, and the precipitate that formed was allowed to settle overnight. The almost clear supernatant was siphoned off and the solids in about 500 ml. volume were centrifuged at 12,000 *g* for 1 hr. The brownish black pellet was suspended in 100 ml. distilled water to give a suspension with a titre of $1{\cdot}5 \times 10^{11}$/ml. containing 35 % of the original phage. This suspension was centrifuged at 12,000 *g* for 15 min. and the supernatant obtained was then centrifuged at 39,000 *g* for 30 min. The pellet obtained was suspended in 10 ml. distilled water to give a suspension with a titre of $1{\cdot}6 \times 10^{11}$/ml. This suspension was adjusted to pH 8 with 1 N-NaOH, and 100 μg./ml. each of RNAse and DNAse were added, followed by incubation for 2 hr. at 37°. The suspension was then centrifuged at 39,000 *g* for 1 hr. and the black pellet was suspended in 1 ml. distilled water giving a titre of $3{\cdot}34 \times 10^{11}$/ml. Although in this purification more phage

could have been recovered by centrifuging at higher speed, the aim was to obtain as clean a fraction as possible.

From this final fraction, 0·5 ml. was added to 2 ml. 0·5 N-perchloric acid and heated at 100° for 20 min., cooled, centrifuged, and the supernatant used for RNA and DNA assays. No RNA was detectable, but there was 350 μg. DNA/ml. phage suspension. Thus this preparation contains $1{\cdot}06 \times 10^{-9}$ μg. DNA/plaque-forming unit. No claim is made that the preparation analysed is pure, but it seems safe to conclude that the nucleic acid of phage P9 is DNA rather than RNA.

The morphology of P9 from the purified preparation was determined by electron microscopy. Most of the particles in shadowed preparations had a head diameter of 50–55 mμ, a tail diameter of 10–12 mμ, and a tail length of 220 mμ. A small, distinct knob could be seen at the tip of the tail, but there was no evidence of tail fibres. In preparations negatively stained with phosphotungstate it was possible to see a thin hole up the centre of the tail which led into the head. Occasional collapsed heads appeared filled with phosphotungstate, and the protein coat of the head could be clearly seen. These particles are quite typical of many DNA phages and bear no resemblance to the streptomycin-sensitive RNA phage of *E. coli* (Loeb & Zinder, 1961). Electron micrographs of phages P1, P4, P10, P13 and P15 revealed that they all had a typical appearance, with large heads and long narrow tails with complete absence of tail fibres.

Minimum inhibitory concentration of streptomycin

The minimum concentration of streptomycin necessary to inhibit P9 replication on plates was about 100 μg./ml. This was approximately the same concentration that was effective with the RNA phage of *Escherichia coli* (Brock, 1962). Since the parent streptomycin-sensitive strain, x13, grows at this concentration of streptomycin, it was possible to show that streptomycin would also inhibit P9 replication in this strain. Thus the streptomycin effect is not restricted to the antibiotic-resistant host, but the use of the antibiotic-resistant host makes it possible to use higher concentrations of streptomycin.

Table 3. *Effect of streptomycin on free* P9 *phage particles*

Time (hr.)	Expt. 1	Inactivation (%)	Time (min.)	Expt. 2	Inactivation (%)
0	$2{\cdot}42 \times 10^9$/ml.	—	0	$1{\cdot}1 \times 10^8$/ml.	—
1	$1{\cdot}26 \times 10^9$/ml.	48	5	$8{\cdot}2 \times 10^5$/ml.	35
2	$1{\cdot}49 \times 10^9$/ml.	38	15	$6{\cdot}5 \times 10^5$/ml.	41
3	$1{\cdot}65 \times 10^9$/ml.	32	60	$2{\cdot}8 \times 10^5$/ml.	75
4	$8{\cdot}5 \times 10^8$/ml.	65	120	$9{\cdot}0 \times 10^4$/ml.	92

1000 μg. streptomycin sulphate/ml. added to a P9 lysate and the titre determined at various times after incubation at 37°.

Action of streptomycin on free phage

It has been known for many years that high concentrations of streptomycin (5000 μg./ml.) will slowly inactivate free T2 particles (Cohen, 1947). To measure the effect of the antibiotic on P9, 1000 μg./ml. was added to a P9 lysate and the titre measured at various times after incubation at 37° (Table 3).

There is only a slow drop in titre over this period and this decrease cannot account for the fact that the antibiotic prevented the appearance of plaques when as many as 10^{10} plaque-forming units/ml. were plated on streptomycin agar.

Action of streptomycin on adsorption of phage P9

Phage P9 was found to adsorb very slowly and this fact complicated the experiments on the effect of streptomycin on adsorption. By reducing the multiplicity of phage: bacterium to a very low level, and by using an adsorption time of 15 min., it was possible to achieve 50–90 % adsorption (Table 4).

Table 4. *Effect of streptomycin on adsorption of phage P9*

	Titre (plaque-forming units/ml.)	Adsorption (%)	Inhibition (%)
Expt. 1			
No cells, no SM	$7{\cdot}9 \times 10^5$	—	—
No cells, SM present	$5{\cdot}2 \times 10^5$	—	—
Cells (5×10^8/ml.), no SM	8×10^4	90	—
Cells (5×10^8/ml.), SM present	3×10^5	42	53
Expt. 2			
No cells, no SM	7×10^5	—	—
No cells, SM present	7×10^5	—	—
Cells (5×10^9/ml.), no SM	$3{\cdot}3 \times 10^4$	53	—
Cells 5×10^9/ml.), SM present	$2{\cdot}3 \times 10^4$	66	0
Cells (5×10^8/ml.), no SM	$8{\cdot}4 \times 10^4$	71	—
Cells (5×10^8/ml.), SM present	$2{\cdot}7 \times 10^5$	49	31

Adsorption time, 15 min., 37°. 1000 μg. streptomycin sulphate/ml. After incubation, cells were removed by centrifugation at 10,000 r.p.m. for 3 min. and the supernatant assayed for phage. Percentage adsorption in the streptomycin series is calculated using as a base-line the tube lacking cells but containing streptomycin.

The greatest inhibition of adsorption by streptomycin is about 50 %. These experiments are complicated by the fact that streptomycin is also able to inactivate free phage (see previous section) and it is not clear whether the adsorption in the streptomycin series should be calculated using a control lacking cells but containing streptomycin, or a control lacking both cells and streptomycin. When a high cell concentration (5×10^9/ml.) is used, streptomycin did not inhibit adsorption. It is possible that streptomycin does not inhibit adsorption by combining with the cells and blocking the receptor sites, but by combining with the virus particle and blocking a specific site on the phage tail. Since the greatest inhibition of adsorption was about 50 %, it seems unlikely that this effect can account for the total antiviral action of the antibiotic.

Influence of streptomycin on replication of phage P9

The influence of streptomycin on replication has been studied in a series of experiments in which one-step growth curves were performed with addition of streptomycin at various times before or after infection. Because of the slow rate of adsorption of P9, high multiplicities were used to achieve significant numbers of infected cells at short adsorption times.

Streptomycin was added before adsorption and then diluted out to allow growth of the control (Fig. 1). On the assumption that the initial phage titre in the chloroform-treated samples represents free phage (in control experiments it could be shown that free phage did occur in the growth tube as a result of unadsorbed phage particles that were trapped in the filter), it is seen that about 99 % of the assayable plaque-forming units were infected centres. In the control the latent period was about 30 min., and the initial burst was about 100. Since adsorption and filtration took about 6–10 min., the actual latent period was between 30 and 40 min. The results do not conform exactly to a one-step growth curve, since the titre continued to rise at a reduced rate after the initial burst, but it is likely that this secondary rise was due to readsorption of released phage on to uninfected cells, probably in the same chain of cocci. Under conditions achieved later (Fig. 2) this secondary rise did not

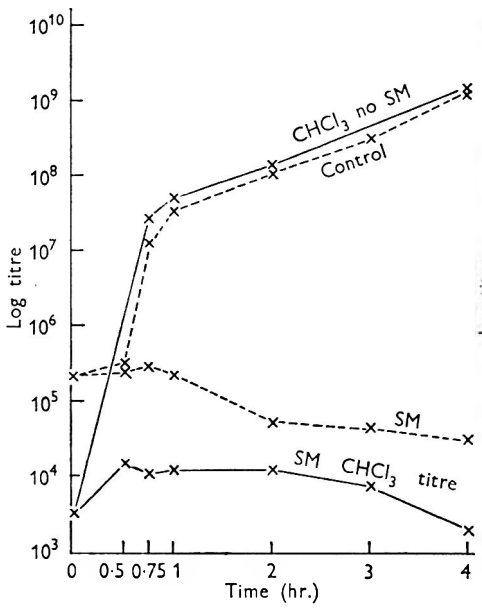

Fig. 1

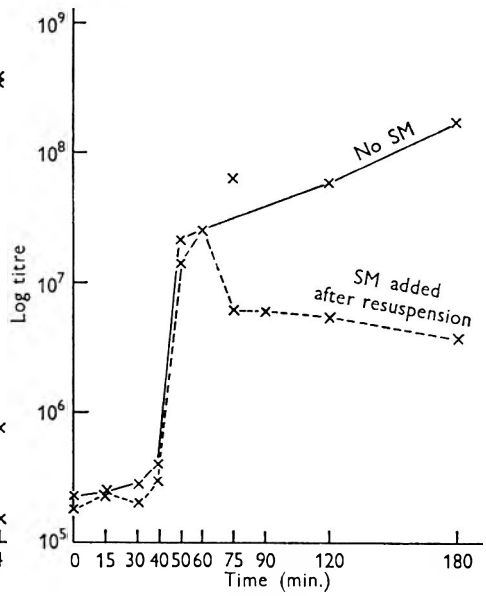

Fig. 2

Fig. 1. Effect of streptomycin (SM) on P9 replication. Cells at 6×10^7/ml. infected in the presence of 1000 μg. streptomycin/ml. with 3×10^9 plaque-forming units/ml., adsorption 5 min. at 37°, then mixture filtered, washed, and resuspended in streptomycin-containing broth. After resuspension, one sample diluted 1/100 in plain medium and one sample diluted 1/100 in streptomycin-containing broth. Zero time is the point when this last dilution was made. Parallel titrations of untreated and chloroform-treated samples.

Fig. 2. Effect of time of addition of streptomycin on P9 replication after adsorption in antibiotic-free medium for 5 min. Cells (6×10^7/ml.) were infected with P9 (1×10^9/ml.) in the absence of streptomycin, incubated for 5 min. at 37°, filtered to remove free phage, diluted 1/100 and resuspended in plain broth. 1000 μg. streptomycin sulphate/ml. was added to one tube immediately after dilution, and phage titres made after incubation at 37°.

occur. The present results (Fig. 1) show that a productive infection could occur when adsorption had taken place in the presence of streptomycin. The latent period was similar to that obtained when the whole growth cycle occurred in the absence

of the antibiotic. As Fig. 1 also shows, streptomycin was able to inhibit replication completely, and the number of infected centres gradually decreased over a 4-hr. period. The fact that the titre of the chloroform samples was at least tenfold lower throughout this period indicates that most of the infected cells retained their phage complement in a form which differed from that of mature phage particles. Since most of these infected cells gave rise to plaques when they were diluted out of the antibiotic, it follows that under the stated conditions many of the phage genomes remained intact throughout the 4-hr. incubation period. The reversibility of the inhibition will be discussed in more detail below. The experiment (Fig. 1) has been repeated a number of times with minor modifications and essentially the same results have been obtained each time. Another experiment indicates that streptomycin inhibition was reversible even after 6 hr. incubation under conditions in which the culture was diluted continuously to keep it in the exponential phase of growth.

Reversibility of streptomycin inhibition

Cells were infected in the presence of 1000 μg. streptomycin/ml. and incubated for 30 min., the normal latent period in the absence of the antibiotic. The suspension was then filtered to remove the antibiotic, suspended in fresh medium, and assayed periodically to determine the latent period. No replication occurred in the presence of the antibiotic and a normal latent period occurred upon removal of the antibiotic. Titrations during the period of streptomycin treatment showed that inhibition of replication was complete and that there was no drop in phage titre. Titres of the chloroform samples showed that replication began almost as soon as the antibiotic was removed.

Effect of time of addition of streptomycin in relation to time of infection

It has been shown above (Fig. 1) that streptomycin can inhibit replication completely when added before infection. A series of experiments were performed to see whether streptomycin would inhibit when added after infection. In the initial series, a 5-min. adsorption time was used, the free phage was removed by filtration, and the antibiotic was added to the infected cells at various times after resuspension. The results in Fig. 2 are in striking contrast to those in Fig. 1. No inhibition was obtained even when the antibiotic was added immediately after resuspension (about 6 min. after initial infection), and similar results were obtained when the antibiotic was added later during the latent period. The latent period and initial burst were identical to that in the control. However, one difference was noted in the period after the initial burst. The control suspension showed a secondary increase in titre (also noted in Fig. 1), while the streptomycin-treated suspension did not show this secondary rise, but showed a slight decrease in titre. Apparently the secondary rise in titre is due to the adsorption of released phage on to unlysed cells and subsequent replication, and this latter replication is inhibited by the antibiotic.

To examine this point further, adsorption time was decreased from 5 to 1 min. and the antibiotic added after removal of free phage (Fig. 3). Under these conditions a quite different result was obtained. The phage titre in the streptomycin-treated sample did not remain constant, as in the earlier experiment (Fig. 1), but decreased

rapidly to about one-tenth the initial value and was not increased by the removal of the streptomycin at any later time. The difference between this experiment and that described in Fig. 1 was that in the latter streptomycin was present before infection, whereas in the former the antibiotic was added shortly after adsorption.

To study this further cells were infected, filtered, washed and resuspended, all in the presence of streptomycin, and then diluted 1/100 to remove the antibiotic. At various times streptomycin was added again and samples plated for infected centres. Under these conditions (Fig. 4) the antibiotic brings about the destruction of infected centres when added at 1, 2, 3 and 4 min. after dilution, but not when added at 6 or 10 min. If the antibiotic is present throughout, there is no loss of infected centres.

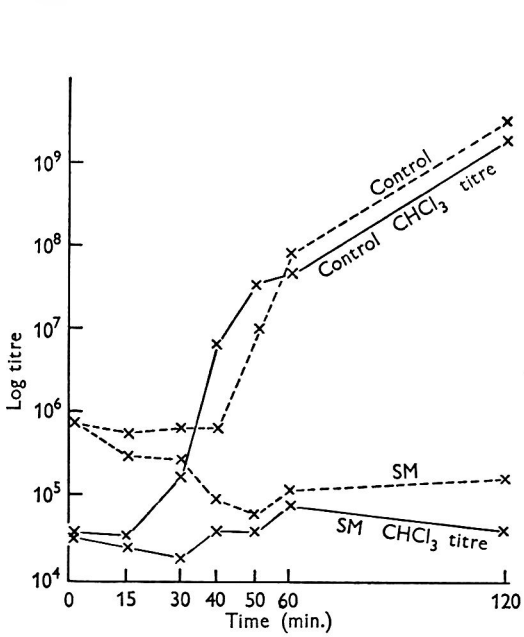

Fig. 3

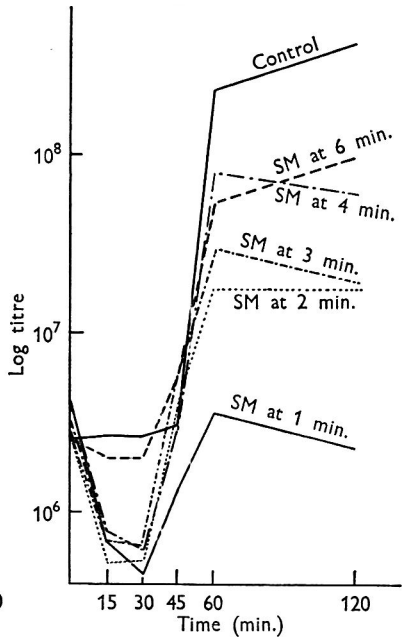

Fig. 4

Fig. 3. Addition of streptomycin after 1 min. adsorption period. Cells ($4{\cdot}8 \times 10^8$ cocci/ml.) were infected with P9 (6×10^9/ml.) for 1 min. in the absence of streptomycin, quickly filtered, washed on the filter and suspended in streptomycin-containing broth at the same cell concentration, and diluted 1/100 in streptomycin-containing broth. A parallel series was run in the complete absence of the antibiotic. Both samples were assayed at various times with chloroform-treated and untreated samples.

Fig. 4. Inactivation of infected centres at different times after infection. Cells ($4{\cdot}8 \times 10^8$ cocci/ml.) were infected with P9 (6×10^9/ml.) in the presence of 1000 μg. streptomycin sulphate/ml., incubated for 5 min. for adsorption, filtered, washed, and resuspended in the presence of the antibiotic. Infected cells were then diluted 1/100 in streptomycin-free medium, incubated, and at stated intervals the antibiotic was added back.

Streptomycin thus apparently has two effects on P9. If added before adsorption, it inhibits injection of the DNA, and the phage-cell complex remains arrested. If adsorption occurs in the absence of the antibiotic, injection of the phage DNA begins, but the DNA remains for 4–5 min. at a site accessible to the antibiotic. The

antibiotic can then combine with the DNA and directly or indirectly bring about its destruction. After the phage DNA has passed to an antibiotic-inaccessible site, the antibiotic has no effect and normal replication occurs.

Nature of the inactivation process

Experiments were performed to analyse the inactivation process brought about by the antibiotic when added during the first 4–5 min. after adsorption. Loss in infected centres begins without a lag upon addition of the antibiotic, and continues at a logarithmic rate for about 30 min., at which time the titre is about ten times less than the initial value. The rate of inactivation depends upon the temperature since it does not occur if the cells are cooled to 0° immediately after the streptomycin is added. Addition of the enzymes deoxyribonuclease or chymotrypsin at concentrations of 200 μg./ml. does not increase the rate of streptomycin-induced inactivation, nor do the enzymes alone cause inactivation. These results do not reveal whether or not the inactivation is enzymic, but do suggest that the process may not be due to a simple precipitation of the DNA by the antibiotic, since this would be expected to occur as readily, or even better, at 0°.

DISCUSSION

Adams (1959) suggested that antibiotics usually prevent phage formation only to the extent of their antibacterial properties. An earlier report of a differential inhibition of phage by streptomycin has been made by Edlinger (1949) for a staphylococcal phage. The brief data reported by him suggest that the mechanism of action of the antibiotic may be similar to that described above, but Edlinger did not carry the investigation this far. Bourke, Robbins & Smith (1952) showed an inhibition by streptomycin of lysis induced in *Escherichia coli* strain B by T2 bacteriophage, but the effect they studied seems to be different from that reported here since Brock (1962) has shown that streptomycin does not reduce the number of plaques formed by T2 on a streptomycin-resistant strain of *E. coli*. Jones (1945) and Cohen (1947) have shown that high concentrations of streptomycin slowly inactivate free phage, but this again is a different phenomenon from that reported here. Graham (1953) showed that streptomycin inhibits superinfection breakdown in T2-infected *E. coli*, but his data indicate that this was because of inhibition of a phage-specific DNAse. Graham & Nelson (1954) showed that streptomycin affected a *Streptococcus lactis* bacteriophage only to the extent that it affected the host bacteria. They did show, however, a phage-specific effect of crystal violet, although in only two of 43 phages tested. Finally Reiter & Oram (1962) have shown that suranim affects a *S. lactis* bacteriophage by inhibiting adsorption.

In the present work it has been shown that a wide variety of bacteriophages attacking various strains of *Streptococcus faecalis*, *S. faecium*, *S. liquefaciens*, and *S. zymogenes* are specifically inhibited by streptomycin. The fact that not all phages are inhibited indicates that the inhibition is a phage-specific, rather than a host-specific phenomenon. Earlier work (Brock, 1962) has shown that in *Escherichia coli* only an RNA phage was affected by the antibiotic.

An analysis of the data in the present paper indicated that streptomycin has two effects. If added before phage adsorption, it seems to inhibit the injection

mechanism, and the phage particle may remain attached to the cell and be carried along through a large number of divisions. This action of the antibiotic is readily and instantly reversible by dilution. If the antibiotic is added shortly after adsorption, or is again added to a culture from which it has just been removed by dilution, it now brings about a rapid inactivation of the phage genome. The rate of inactivation depends upon the temperature so it may be enzymatic, although added DNAse or chymotrypsin had no effect. The details of the inactivation process remain to be worked out.

If the streptomycin is again added 6 min. after injection begins, it has no effect on phage replication, and a burst occurs after the normal latent period of about 35 min. If the antibiotic is added at any time between 1 and 4 min. after injection begins, inactivation occurs. It thus seems reasonable to conclude that the time for complete injection is 5–6 min. Once injection has occurred, the phage genome is in a site inaccessible to the antibiotic. Recent work (Brock & Wooley, 1963) using high-speed blending has confirmed that streptomycin inhibits injection.

Bradley & Kay (1960) have studied the morphology of a variety of bacteriophages, using the phosphotungstic acid negative-staining technique. They have shown that the long-tailed phages fall into two groups. In one, the phages have contractile sheaths probably associated with the injection mechanism. In the other group, contractile sheaths are lacking, and another means of injection probably exists. Phage P9 has been studied in detail under the electron microscope and phages P1, P4, P10, P13 and P15 have been examined briefly. All are long-tailed phages with no evidence of contractile sheaths. Phage P3, which is streptomycin-resistant, has a head size of about 60 mμ and a tail size of about 200 mμ, and thus is not grossly different from the streptomycin-sensitive phages.

In addition to its usefulness in analysing the mode of injection of a phage nucleic acid, streptomycin can be used to advantage in classifying various bacteriophages. Once streptomycin-resistant host mutants are available, the determination of antibiotic sensitivity is a simple task. It would be very useful to know the streptomycin sensitivity of a wide variety of phages, especially for Gram-positive bacteria. At least one staphylococcal phage is streptomycin-sensitive (Edlinger, 1949).

If streptomycin sensitivity is associated with some aspect of the injection mechanism, this characteristic might be used as a genetic marker of high selective value. However, attempts to isolate streptomycin-resistant mutants of phage P9 have been unsuccessful. It has also been impossible to demonstrate recombination between P3 (streptomycin-resistant) and P9 (streptomycin-sensitive) but since these two phages are obviously quite unrelated, this result is not surprising.

Finally, this work presents a model of how an antiviral compound might work. Since the host cells are completely resistant to the antibiotic, it is hard to imagine how an antiviral agent might inhibit by attacking some part of the machinery which is essential for both bacterial and viral reproduction. Thus it is significant that the site of attack of the antibiotic is on a phase of reproduction before the phage genome is integrated into the cell. Bacterial viruses have probably evolved special mechanisms for penetrating the rigid bacterial cell wall, and this probably explains the universal occurrence of tails in phage particles which have large heads. Only the very small phages, such as ØX174, MS–2 and f2 seem to lack tails, but these phages are so small that they can probably pass directly through the cell-wall mesh and

attach themselves to the cell membrane. Thus in all large bacterial viruses, an injection mechanism for the DNA is essential. The question arises whether animal viruses also have injection mechanisms for penetrating cells, or whether these viruses enter through an activity of the animal cell itself, such as pinocytosis or phagocytosis. Apparently little is known about the mechanism of penetration of animal viruses (Hirst, 1959), and an investigation of this process in a variety of cases might provide important clues for chemotherapy. It should be pointed out that successful chemotherapy can occur even if only a small portion of the virus life cycle is affected, since virus replication is a progressive process in which the particles that leave one cell attack other cells. Thus streptomycin, although affecting only the first four minutes of the P9 life cycle, can completely inhibit the formation of plaques even when more than 10^{10} infectious units are put on a plate. This is indeed successful and impressive chemotherapy.

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The Physiology and Nutrition of some Aquatic Hyphomycetes

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SUMMARY

The nutrition and physiology of some aquatic Hyphomycetes were examined. Optimum growth temperatures were below those usually encountered amongst aquatic moulds. Several species utilized almost all the carbohydrates tested and most gave a molar growth yield between 80 and 95 mg. mycelial dry weight/m-mole carbohydrate utilized. Of the vitamins tested none appeared to be essential nutrients for most species, though some were stimulatory. Nitrate and ammonium ions both served as adequate nitrogen sources, with a preference for the latter. The ways in which aquatic Hyphomycetes may have nutritional advantages over their possible ecological competitors are discussed.

INTRODUCTION

Ingold and collaborators (see Peterson, 1962) drew attention to a distinctive group of aquatic Fungi Imperfecti of world-wide distribution, of which about 60 species have been described, and which live on decaying leaves of deciduous trees in rapidly flowing streams. The mycelia infest the tissue of the decaying leaves and produce conidiophores which project into the water. The organisms are not confined to this habitat. They have been found growing on beech roots (Waid, 1954), the leaf carpet of woods (Scourfield, 1940), pine bark (Saccardo, 1876, 1880), and Ranzoni (1953) stated that maple samaras, peach stones, fern rhizomes, small twigs and decomposing sporophylls of Isoetes, onion scales and paper serve as well as leaves as substrata for these organisms. The aquatic habitat, however, is certainly a characteristic one where they can regularly be found. Although our knowledge of the morphology and conidial development of these fungi is adequate, comparatively little is known about their ecology and almost nothing about their nutrition and physiology. Ranzoni (1953) investigated the carbon nutrition and vitamin requirements of two species of Anguillospora, but apart from these observations there appears to be no published work. In an attempt to fill this gap in knowledge an investigation was made of the nutrition and physiology of some common species of this group. It was hoped that the results of this investigation might throw light on the ecology, and explain in part the preference for the rather unusual habitat to which these fungi have become adapted.

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METHODS

Pure cultures were started from single spore isolates and maintained at 15° in Petri dishes containing malt extract agar (see below). The method of isolation of single spores and the stimulation of spore production from mature culture was as described by Webster (1959). The experiments concerned with carbon and vitamin nutrition involved the use of 1 ml. of a suspension of washed spores as inocula. Sterile spores were produced by aerating a suspension of mature mycelium under sterile conditions, then washing by centrifuging at 9000 rev./min. (5000*g*) under sterile conditions with several changes of sterile distilled water.

Media. The following media were used throughout the work. (1) Malt agar for culture maintenance; 'Oxoid' malt extract 16 g., agar 20 g., distilled water 1 l. (2) Liquid malt extract: a solution of malt extract in distilled water (16 g./l.), repeatedly filtered through Whatman's no. 1 filter paper. (3) Carbon nutrition was examined by using the following medium (based on Ranzoni, 1953): carbohydrate, 4·0 g.; KH_2PO_4, 1·0 g.: $MgSO_47H_2O$, 0·2 g.; $FeCl_3$ (commercial), 0·02 g.; Difco yeast extract, 1·0 g.; distilled water, 1 l. All compounds were obtained from British Drug Houses (Poole, Dorset) and were 'Analar' unless otherwise stated. This medium is referred to below as 'Ranzoni Medium' and was usually sterilized by Seitz filtration. When starch and methylcellulose were used, sterilization was by autoclaving at 115° for 10 min. (4) Basal medium consisted of Ranzoni medium without carbohydrate. (5) Vitamin-free medium used in growth factor experiments was Ranzoni medium, but with the yeast extract replaced by 2 g. asparagine. Vitamins were added as required. (6) Nitrogen nutrition was examined in Ranzoni medium, but with the yeast extract replaced by biotin + pantothenic acid (each 5 μg./l.) and a nitrogen source of potassium nitrate (1·34 g./l.) or ammonium sulphate (0·88 g./l.). At these concentrations each salt contains the amount of nitrogen in 1 g. asparagine, which was earlier found to be a suitable concentration and source. The ammonium sulphate cultures were adjusted to pH 5·6 at each dry-weight determination.

Cultures were incubated in 20 ml. medium in 100 ml. conical flasks. For carbon, vitamin and nitrogen nutrition studies a temperature of 15° was selected. Shaken cultures (90–100 oscillations/min. with a throw of 2 in.) gave maximum yield. Mycelial dry weight was determined by filtering off the medium by suction through previously weighed Whatman no. 1 filter papers, drying the mycelial mat for 4 hr. at 125°, cooling in a desiccator and reweighing.

Estimation of residual soluble carbohydrates. The anthrone technique described by McCready, Guggolz, Silviera & Owens (1950) was used, but modified to avoid an unexplained white precipitation of the reagent on dilution with used media. A sample (1 ml.) of medium filtered through sintered glass was added slowly to 5 ml. reagent in iced water, then boiled for 2 min. only and cooled for 30 min. at 5°. The blue colour was then measured by an E.E.L. colorimeter using a number 608 light filter. This method was used for all the carbohydrates tested except starch, which was measured by a method also described by McCready *et al.* (1950).

Analysis of residual nitrate and ammonium ion. A Markham still was used for this purpose. After blanks were estimated, ammonium and nitrate ion concentrations in a medium were determined. Ammonium ion concentration was first determined

with excess strong sodium hydroxide, and this was followed by Devada's alloy to find the nitrate concentration. The ammonia distilled over in each case was collected in flasks containing 2 % boric acid with methyl red and bromocresol green indicators.

RESULTS

Optimum temperature. If aquatic Hyphomycetes can metabolize normally at moderately low temperatures, they may be able to compete ecologically with other less well-adapted fungi. To test this hypothesis the optimum temperatures for the growth of eight aquatic Hyphomycetes were determined. The medium used was liquid malt extract and growth was measured as dry weight. The fungi were incubated at 5–30° in 5° intervals in shaken culture. Several readings were taken, those after 11 days of incubation being found most suitable for optimum temperature determination. The results are as follows: *Articulospora tetracladia*, 20–25°; *Flagellospora penicillioides*, 25°; *F. curvula*, 15°; *Lemonniera aquatica*, 20°; *Tricladium splendens*, 15°; *T. gracile*, 20°; *Heliscus lugdunensis*, 20–25°; *Tricellula aquatica*, 10°. Phycomycetes, some aquatic, have higher optimum temperatures than the aquatic Hyphomycetes shown above (Thornton, 1962; Tomlinson, 1952; Roth, 1935; Cantino, 1949).

Carbon nutrition. The utilization of several carbohydrates by some aquatic Hyphomycetes was tested by growing them in shaken culture in Ranzoni medium.

The carbohydrates tested included all those previously identified in leaf litter from alder, oak, beech and birch. *Articulospora tetracladia* was used to determine the yeast extract concentration needed for minimal supply of all growth factors.

For quantitative expression of the results the molar growth yield was adopted (Bauchop & Elsden, 1960). It is defined as:

Dry wt. organism./mmole substrate utilized.

If such yields are to have comparative value it is essential to choose a system in which falling carbohydrate concentration is the factor which first limits growth. If other factors (availability of oxygen, vitamin or trace metal) limit growth first, then an apparently inefficient utilization of the carbohydrate would be found, probably with a build-up of intermediate metabolic products. The apparent inefficiency would in this case be false. It was therefore necessary to determine the concentration of carbohydrates which satisfies the above condition.

The qualitative relationship between the amount of growth of a micro-organism and its nutrient supply was first stressed by Monod (1942) for bacteria. In order to find the appropriate concentration at which carbohydrate should be supplied, preliminary experiments were carried out. They determined the range of concentration within which, for a particular medium, there is a linear relationship between amount of carbohydrate supplied and the dry weight of yield of organism. *Articulospora tetracladia* was grown at various glucose concentrations in Ranzoni medium and incubated statically or shaken at 15°. Figure 1 shows that, in shaken culture, a direct relationship between mycelial yield and glucose concentration existed for this medium at concentrations between 2 and 8 g. glucose/l.; 4 g./l. was chosen for subsequent work. The graph also shows the inhibitory effect of high glucose concentration in standing culture.

The assumption was made that the physiology of *Articulospora tetracladia* and the other fungi to be examined did not differ appreciably with respect to glucose metabolism and detailed studies of suitable carbohydrate concentrations were not made for other fungi. Residual carbohydrate was analysed in all experiments so that failure to achieve complete utilization of glucose would be detected.

The yield of aquatic Hyphomycetes grown on various carbohydrates. The growth of several moulds with the following carbohydrates was examined: glucose, fructose, maltose, cellobiose, xylose, starch, sucrose, methylcellulose. The carbohydrates were supplied at 4 g./l., which gave media containing similar amounts of glucose equivalent, regardless of the carbohydrate used. The pH value of the cultures throughout the experiments was fairly constant. Increasing mycelial dry weights were plotted

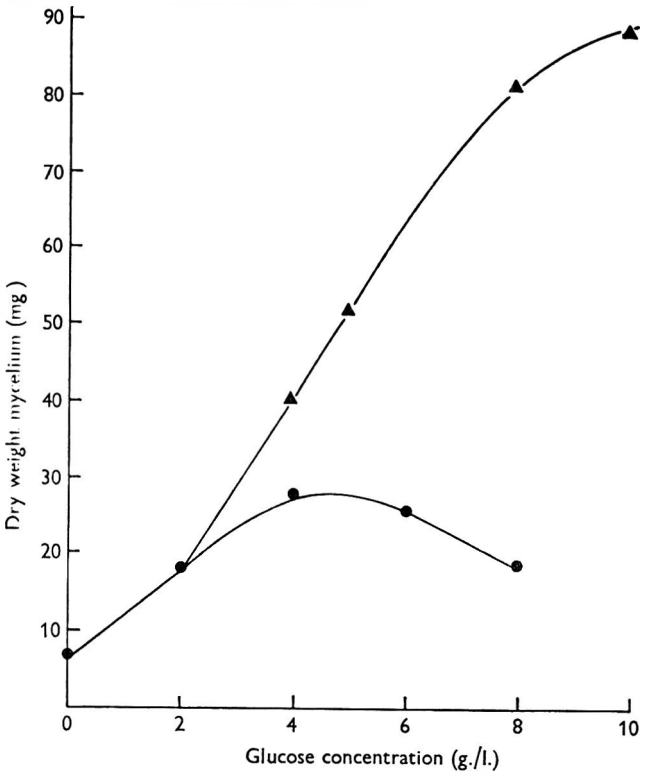

Fig. 1. The effect of shaken and static culture on the dry weight of *Articulospora tetracladia* at different glucose concentrations. ▲, Shaken culture; ●, static culture.

against carbohydrate concentration, which usually fell to zero. Sometimes half-maximum dry weight of organism was obtained when almost complete utilization of carbohydrate was recorded, suggesting that an extracellular breakdown of the hexose molecule might have occurred before utilization. The yields are expressed in terms of molar growth yields (Table 1). No growth was observed on methylcellulose.

The vitamin requirements of some species of aquatic Hyphomycetes. Preliminary experiments showed that the maximum yield of *Articulospora tetracladia* was not affected by the addition of certain growth factors to the basal medium, although pantothenic acid caused a shorter lag. The factors added were: biotin, 5 μg./l.;

thiamine, 100 μg./l.; inositol 5 mg./l; pyridoxine, 100 μg./l.; nicotinamide, 50 μg./l.; riboflavin, 5 μg./l.; pantothenic acid, 5 μg./l.; folic acid, 5 μg./l.; *p*-amino benzoic acid, 5 μg./l.; ascorbic acid, 5 μg./l.

Two test organisms known to require, respectively, the thiazole moiety of thiamine (*Rhodotorula rubra*) and biotin (*Sordaria fimicola*) were used to examine the purity of the medium. Neither species grew on the basal medium alone but was vigorous when thiamine and biotin were added. The medium was therefore considered free from biotin and thiazole or thiamine. Increasing the number of washings of spores for inocula did not affect the results. Experiments were then carried out

Table 1. *The molar growth yields of some aquatic Hyphomycetes on various carbon sources*

Species	Carbohydrate						
	Glucose	Fructose	Sucrose	Maltose	Xylose	Cellobiose	Starch
Articulospora tetracladia	81·7*	83·0	85·6	68·0	80·0	62·1	81·3
Flagellospora penicillioides	72·2	68·4	94·4	84·0	78·2	89·9	—
Heliscus lugdunensis	85·0	97·0	111·5	89·0	79·6	89·5	73·4
Tricladium angulatum	97·0	107·0	80·1	87·5	81·6	104·5	87·5
T. splendens	99·5	96·6	111·5	86·5	89·4	94·0	83·0
Tetracladium setigerum	87·5	86·0	87·3	84·0	78·2	89·9	59·7
Varicosporium elodeae	79·4	82·4	91·0	80·5	69·6	89·4	82·9
Volucrispora aurantiaca	87·4	89·9	91·0	94·8	95·5	107·0	—
Pythium ultimum	84·1	—	—	—	—	—	—
Saprolegnia litoralis	93·0	—	—	—	—	—	—
Dictyuchus sterile	89·4	—	—	—	—	—	—
Sordaria fimicola	83·5	—	—	—	—	—	—
Merulius lacrymans	72·4	—	—	—	—	—	—

* Molar growth yield is expressed as mg. dry wt. mycelium produced per millimole of carbohydrate utilized. Carbohydrate was supplied to above cultures at a concentration of 4 g./l. medium. Incubation was in shaken culture at 15°.

Table 2. *The vitamin requirements of some aquatic Hyphomycetes*

	Requirement	Concentration/l.
(*a*) Prototrophic		
Flagellospora penicillioides	—	—
Tricladium splendens	—	—
Heliscus lugdunensis	—	—
(*b*) Partially auxotrophic		
Articulospora tetracladia	Pantothenic acid	5 μg.
Varicosporium elodeae	Biotin	5 μg.
(*c*) Auxotrophic		
Volucrispora aurantiaca	Biotin (strong requirement)	5 μg.
Tricladium angulatum	Biotin or Pantothenic acid or Inositol	5 μg.

with several other species, vitamins being supplied singly. Several species of aquatic Hyphomycetes had no detectable vitamin requirements, whilst in others the lag period was shortened in the presence of certain vitamins, but all cultures eventually reached the same dry weights. Only one species (*Volucrispora aurantiaca*) had an absolute requirement, namely for biotin (Table 2). The requirements for *Tricladium angulatum* were rather unusual. Each of biotin, panthothenic acid or

inositol caused a maximum yield. A similar effect was observed by Emery, McLeod & Robinson (1946) using *Kloeckera brevis*, which required thiamine or inositol.

The inorganic nitrogen nutrition of some aquatic Hyphomycetes. Thresh, Beale & Suckling (1949) showed that most streams on Coal Measures, Millstone Grit and Sandstone contained small concentrations of ammonium and nitrate ions. These might form nitrogen sources for aquatic Hyphomycetes. Thresh *et al.* (1949) showed that nitrite was only found in sewage effluent and nitrite will not be discussed here.

Table 3 records the maximum dry weights of organism found with nitrate or ammonium ions as nitrogen sources. The standard deviation for all results lay between $\pm 0{\cdot}8$ and $\pm 1{\cdot}2$. The average pH value for growth with nitrate was 7·4 and for growth with ammonium ion was 4·4. This would have been considerably lower but for pH adjustment with 4 % NaOH.

Table 3. *The yields (mg.) of aquatic Hyphomycetes on nitrate and ammonium ions*

Species	14 days incubation yield/flask, nitrate	11 days incubation yield/flask, ammonium	Controls (no nitrogen)
Articulospora tetracladia	28·6	31·7	1·6
Varicosporium elodeae	29·4	38·8	1·4
Tricladium splendens	33·7	38·9	1·7
Flagellospora penicillioides	29·5	28·9	1·8
T. angulatum	22·3	26·3	0·6
Heliscus lugdunensis	31·7	36·2	0
Volucrispora aurantiaca	Trace	0	0

Table 4. *Percentage utilization of nitrate and ammonium ions by aquatic Hyphomycetes on the 6th and 13th days of incubation at a temperature of* 15°

The medium contained glucose, 4·0 g.; KH_2PO_4, 1·0 g.; $MgSO_4$, 0·2 g.; $FeCl_3$, 0·02 g.; biotin 5 μg.; pantothenic acid, 5 μg.; distilled water, 1 l.

Species	6th day		13th day	
	% NH_4 utilized	% NO_3 utilized	% NH_4 utilized	% NO_3 utilized
Articulospora tetracladia	99·4	67·4	99·4	96·1
Varicosporium elodeae	82·3	21·0	99·4	98·0
Tricladium splendens	74·1	22·4	99·4	98·9
T. angulatum	34·9	9·3	99·4	98·4
Flagellospora penicillioides	79·1	26·4	99·4	99·4
Heliscus lugdunensis	65·2	14·5	97·5	99·4

Because the media still contained residual nitrogen at the end of the experiments, nitrogen starvation could not have been responsible for stopping growth.

Preferential utilization of inorganic nitrogen. The previous experiments indicated that a greater yield of organism was attained with ammonium ion than with nitrate ion. A test was therefore made to see whether a preference for nitrate or ammonium ion existed. The nitrogen source was a mixture of ammonium sulphate and potassium nitrate, each containing an equal weight of nitrogen, so that the two sources together equalled the weight of nitrogen in 1 g. asparagine. Table 4 shows analyses

made on the 6th and 13th days of incubation, of the ammonium and nitrate % utilization for all species except *Volucrispora aurantiaca*, which used neither source of nitrogen.

DISCUSSION

The results obtained here for aquatic Hyphomycetes, when compared with the optima for some Phycomycetes suggest that the Hyphomycetes may have a physiological advantage over their environmental competitors because of the rather low temperatures that they require for optimal growth. A further advantage may be the low optimum temperatures needed for sporulation, as found in some preliminary experiments.

The aquatic Hyphomycetes can use a wide range of simple and polymer carbohydrates, most of which occur naturally in abscissed leaves, but the Phycomycetes, a comparable group, are more specific in their nutritional requirements. For instance *Chytridium* sp. cannot use maltose, xylose, sucrose or cellulose; *Macrochytrium botryoides* cannot use fructose, sucrose or cellulose (Crasemann, 1954); *Allomyces arbuscula* and *A. cystogenus* can only use glucose, maltose, and mannose (Ingraham & Emerson, 1954). However, the method of aeration used by Ingraham & Emerson was thought by Professor S. R. Elsden (private communication) to be inadequate. He assumed 50 % oxidation of glucose and 50 % conversion to cellular material, from molar growth yield results; thus an organism in 50 ml. of medium containing glucose at 5 g./l. when fully respired would require approximately 445 ml. of air. Ingraham & Emerson's conditions (125 ml. flasks, shaken) allowed only 75 ml. air, the growth rate being limited by the rate of diffusion of oxygen through the cotton-wool plug. Other examples of fungi unable to use many carbohydrates are provided by Ajello (1948), Cantino (1949) and Lilley & Barnett (1953). The aquatic Hyphomycetes should therefore have a nutritional advantage over some of their environmental competitors with respect to carbohydrate use.

The most frequently encountered values of molar growth yield fell between 80 and 95 mg. mycelium/mmole carbohydrate utilized. Similar yields were recorded with *Escherichia coli* and *Arthrobacter globiformis* (Morris, 1960), *E. coli* strain 4071 (Whitaker, 1962), *Aspergillus niger* (Terroine & Wurmser, 1922), and *Penicillium chrysogenum* (Pirt & Callow, 1960).

The only requirement for vitamin was by *Volucrispora aurantiaca* for biotin, but this organism may not be a true aquatic Hyphomycete (Haskins, 1958; Ingold, 1959). The only other effect was a shortening of the lag period by pantothenic acid with *Articulospora tetracladia*, an unusual requirement in fungi. It may be suggested that aquatic Hyphomycetes might have some nutritional advantage over the other aquatic moulds by virtue of their high degree of vitamin autotrophism.

The ease with which the aquatic Hyphomycetes utilize inorganic nitrogen (except Volucrispora) is not a common feature of other aquatic moulds. Whiffen (1945) recorded no growth of Saprolegniaceous moulds on $(NH_4)_2SO_4$ or KNO_3, but adequate growth on organic nitrogen sources. Reischer (1951) recorded poor growth of six species of Achlya as well as Protoachlya, Isoachlya, *Saprolegnia delicia* and Thraustotheca on nitrate. The aquatic Hyphomycetes may, therefore, benefit more from the nitrate and ammonium ions leached from the soil into streams than do other aquatic moulds.

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Effect of Environmental Conditions on the NADP-Specific Glutamic Acid Dehydrogenase in *Neurospora crassa*

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SUMMARY

The chemical form and quantity of nitrogen supplied during the growth of wild-type *Neurospora crassa* mycelia had a significant effect on total protein synthesis and NADP-specific glutamate dehydrogenase (NADP-GD) specific activity. Both NADP-GD specific activity and protein yields were high when NH_4^+ was the sole nitrogen source. At NH_4^+ concentrations up to the optimal for protein synthesis, NADP-GD production and protein synthesis were proportional; at higher concentrations NADP-GD specific activity decreased disproportionately. Glutamate alone, more markedly glutamate + NH_4^+, or a mixture of amino acids (e.g. sodium caseinate), similarly depressed NADP-GD specific activity. These data support the contention that excess nitrogen in some form acts as a specific NADP-GD repressor. Evidence is presented from experiments with nitrogen-starved mycelia that low concentrations of NH_4^+ *per se* may act to de-repress NADP-GD production. Under conditions of early nitrogen starvation, followed by a short growth period after NH_4^+ supplementation, more than 1 % of the soluble protein in a heat-treated extract was found to be NADP-GD.

INTRODUCTION

In Neurospora significant modification of enzyme production in response to environmental changes has been demonstrated in only a few instances (Fincham & Boulter, 1956; Horowitz, Fling, MacLeod & Watanabe, 1961; Yura & Vogel, 1959; Kinsky, 1961; Turian, 1961; Lester, 1961). In contrast, the formation of inducible enzymes in bacteria has been found in nearly every case investigated. It is not known whether this apparent discrepancy between filamentous fungi and bacteria reflects fundamental differences in regulatory metabolic mechanisms or methodological difficulties in studying enzyme regulation in the filamentous fungi. Fincham (1962) and Barratt (1961) have reported the purification from mycelia of *Neurospora crassa* of a NADP-specific glutamate dehydrogenase (NADP-GD). Nicholas & Mabey (1960) reported the occurrence of a glutamate dehydrogenase (GD) from Neurospora which utilizes either diphosphopyridine nucleotide (NAD) or triphosphopyridine nucleotide (NADP) as a coenzyme. Recently two glutamate dehydrogenases, one specific for NAD and the other specific for NADP, have been isolated (Sanwal & Lata, 1961*a*, *b*), and these authors have reported effects of environmental conditions on the concurrent regulation of these two enzymes in Neurospora.

The present paper reports the effects of certain environmental conditions on NADP-GD production and specific activity in Neurospora. Specifically, the effects of different nitrogen sources and the time of nitrogen supplementation are reported.

METHODS

Organisms. The following strains of *Neurospora crassa* were used: wild-types-STA 4 (an asexual derivative of St Lawrence 74A), 1A, SY 7A, SY 4A. All these strains were obtained from the Fungal Genetics Stock Center, Dartmouth College, Hanover, New Hampshire, U.S.A.

Chemicals. α-Ketoglutaric acid and reduced triphosphopyridine nucleotide ($NADPH_2$) were obtained from Sigma Chemical Company, St Louis, Missouri, U.S.A. (95% pure; type I), the amino acids from the California Corporation for Biochemical Research, Los Angeles, California, U.S.A. and sodium caseinate from Nutritional Biochemical Corporation, Cleveland, Ohio, U.S.A.

Assay of NADP-GD. Assays were carried out by following the oxidation of reduced triphosphopyridine nucleotide ($NADPH_2$) in a quartz spectrophotometer cell of 1 cm. light path in a Beckman Model DU or a Cary Model 14 spectrophotometer at 340 mμ at room temperature. To a 4 ml. cell were added: 2·1 ml. 0·2 M-2-amino-2-hydroxymethylpropane-1,3-diol (tris) buffer (pH 7·8); 0·2 ml. 0·167 M-α-ketoglutarate (pH 7·0), 0·15 ml. 3·0 M-NH_4Cl in tris buffer; 0·16 ml. of $1{\cdot}2 \times 10^{-3}$ M-$NADPH_2$ in tris buffer (0·16 ml. was sufficient to give a change in optical density (OD) of 0·4). The reaction was initiated by the addition of 0·1 ml. of enzyme solution. Enzyme concentrations were kept sufficiently low to give a linear reaction curve from 0 to 6 min.; in general one unit or less of NADP-GD was used in each assay. When crude enzyme preparations were used the reaction was initiated with α-ketoglutarate and non-specific $NADPH_2$ oxidation was subtracted from the rate obtained. In most experiments heat-treated fractions were used; these did not show any $NADPH_2$ oxidation before substrate addition. A unit of enzyme activity was defined as the change in optical density at E_{340} of 0·02/min. Based on data to be published elsewhere, pure NADP-GD has a specific activity of about 25,000 units/mg. protein when assayed at 25°.

Protein determinations. Protein determinations were made on the soluble proteins by using the Folin phenol method of Lowry, Rosebrough, Farr & Randall (1951). Specific activity is expressed as units of enzyme activity/mg. protein.

Culture media. Four types of basal media were used: Fries medium (Beadle & Tatum, 1945); medium N (Vogel & Bonner, 1956); the basal nitrogen-free medium of Fincham (1954) supplemented with trace elements (Westergaard & Mitchell, 1947) and designated as medium F in the present paper; Wainwright's medium for producing conidia (Wainwright, 1959) supplemented with the above trace elements and designated as medium W in the present paper. The carbon source of medium W was modified by replacing one half of the sucrose with glycerol (i.e. final concentration 1% (v/v) glycerol).

Preparation of large batches of conidia. Maximal NADP-GD specific activity occurs shortly after conidial germination. Therefore to obtain significant quantities of protein, large conidial inocula became essential. Conidia were grown according to the method of Wainwright (1959) with the following modifications. Inoculated

Fernbach flasks, containing 250 ml. modified medium W, were incubated at 34° in an upright position for 24 hr., then inverted and incubated for a second 24 hr. period. To induce maximal conidiation the flasks were transferred to room temperature in the light, inverted and aerated with a gentle stream of sterile humidified air for 3–4 days (about half the time necessary for maximal conidiation reported by Wainwright, 1959). Conidia were harvested in a 0·1 % (v/v) sterile Tween 80 solution, loosened with a sterile nylon test-tube brush, filtered through a double layer of sterile gauze to remove mycelial fragments, centrifuged at 3000*g* for 10 min., and resuspended in sterile water. This procedure routinely yielded from each flask 3 to 6×10^{10} conidia wet weight about 3·2 g. (dried by vacuum suction on Whatman no. 4 filter paper).

Conditions for growth. All mycelia used in these experiments were grown in aerated submerged cultures. No difference in total NADP-GD activity or specific activity was observed as between aeration on a shaker and this submerged forced aeration.

Preparation of extracts. Mycelia were harvested by vacuum filtration through S. and S. Sharkskin (Carl Schleicher and Schuell Company, Keene, New Hampshire, U.S.A.) filter paper, washed with distilled water and phosphate buffer (0·1 M; pH 7·0), frozen and stored. Frozen mycelium was ground in a Waring blender in cold 0·1 M-phosphate buffer (pH 7·0) until uniformly homogenized, then transferred to a Virtis '45' homogenizer (The Virtis Company, Inc., Yonkers, New York, U.S.A.) and homogenized at full speed for 20 min. in an ice bath. Routinely, the final homogenate was heated at 53° for 30 min., cooled, centrifuged at 12,000*g* and the supernatant fluid assayed.

RESULTS

Effect of chemical form of nitrogen

In vivo in Neurospora, NADP-GD functions in the reductive amination of α-keto acids since *am* strains, known to be deficient in NADP-GD do not grow in absence of transaminable α-amino nitrogen (up to 3 days; Fincham, 1950). Consequently, the chemical form of nitrogen supplied might be expected to exert a regulatory effect. Neurospora mycelia were grown in media containing the following nitrogen sources: (*a*) NH_4^+; (*b*) one half NH_4^+ and one half NO_3^- (standard minimal medium N); (*c*) readily transaminable α-amino nitrogen; (*d*) amino nitrogen not transaminable. The specific NADP-GD activities found under these growth conditions are given in Table 1. Twenty-five-fold differences in specific activity and total NADP-GD production were observed, but not under the same experimental conditions. Both NADP-GD-specific activity and protein yields were high when nitrogen was supplied as NH_4^+. Specific activity was the highest but growth and protein synthesis were minimal when the nitrogen was supplied in a poorly available form, e.g. glycine, serine, lysine (which Neurospora cannot transaminate; Fincham, 1951). The mycelia from these three flasks were pooled. Protein synthesis was high but specific NADP-GD activity was very low when nitrogen was supplied as a mixture of readily available amino acids (e.g. sodium caseinate). When α-amino nitrogen was supplied as glutamate, protein synthesis was limited, NADP-GD-specific activity was high and increased with decreasing glutamate concentrations. Glutamate or some derived metabolite is a repressor of NADP-GD. However,

other amino acids or their derived metabolites can also act as repressors, since hydrolysed casein decreases NADP-GD production much below that caused by a comparable concentration of glutamate. When one half of NH_4^+ was replaced by nitrate ion (standard medium N) protein synthesis and specific activity were low.

Table 1. *Effect of nitrogen source*

Neurospora mycelium grown in 50 ml. culture medium containing 2% (w/v) sucrose for 46 hr. at 18° on rotary shaker. Inoculum: 6 drops of unfiltered conidial suspension of Neurospora strain STA 4. Assays on non-heat treated mycelial extracts.
Molarity (M) is expressed in terms of nitrogen.

Culture medium		Supplement		Total protein (mg.)	NADP-GD specific activity (u./mg.)
Type	Final pH	Compound	Nitrogen (M)		
F*	6·8	Ammonium tartrate	0·032	35·0	139
F	4·0	Ammonium tartrate	0·065	25·5	172
F	4·5	Ammonium tartrate	0·260	21·5	9
F	6·2	Glutamate†	0·012	7·4	167
F	6·1	Glutamate	0·025	6·2	121
F	6·3	Glutamate	0·100	9·6	109
F	5·6	Sodium caseinate	0·086‡	25·2	20
F	5·6	Glycine	0·050	5·2§	242
F	5·3	Serine	0·037		
F	5·3	Lysine	0·140		
N	5·2	None	0·050‖	8·2	45

* Initial pH value of medium F = pH 5·65.
† Neutralized to pH 5·0 with NaOH.
‡ Assumes molecular weight of sodium caseinate to be 150 and nitrogen content to be 16%.
§ For the three flasks.
‖ Medium N contain nitrogen as 0·025M-NH_4^+ and 0·025M-NO_3^-.

Effect of ammonium ion concentration

As described above, the different concentrations of NH_4^+, supplied as NH_4 tartrate, resulted in very different specific activities. Therefore, the regulatory effect of different concentrations of NH_4^+ was investigated. A thirty-eight-fold difference in total activity, a fourfold difference in total protein and nearly a ten-fold difference in specific activity was found over the range of nitrogen concentrations tested (Fig. 1). These marked differences were not accompanied by similar differences in the wet weight yields of mycelium. The optimal concentration of NH_4^+ was 0·065M with respect to nitrogen for highest NADP-GD-specific activity and production. These results are consistent with a regulatory effect of NH_4^+ on NADP-GD yield.

Post-growth nitrogen starvation and its reversal

Ammonium ions exert a regulatory effect: a low NH_4^+ concentration results in high protein and NADP-GD-specific activity. Since any further decrease of NH_4^+ concentration markedly limited growth, post-growth nitrogen starvation might provide similar conditions of extreme NH_4^+ limitation. Two-day mycelia were washed in phosphate buffer, transferred to nitrogen-free medium F and incubated for a further 2 days. The total NADP-GD production and specific activity doubled (Table 2). However, when the nitrogen starvation was prolonged for 6 days, the specific activity remained high but general protein catabolism occurred. More

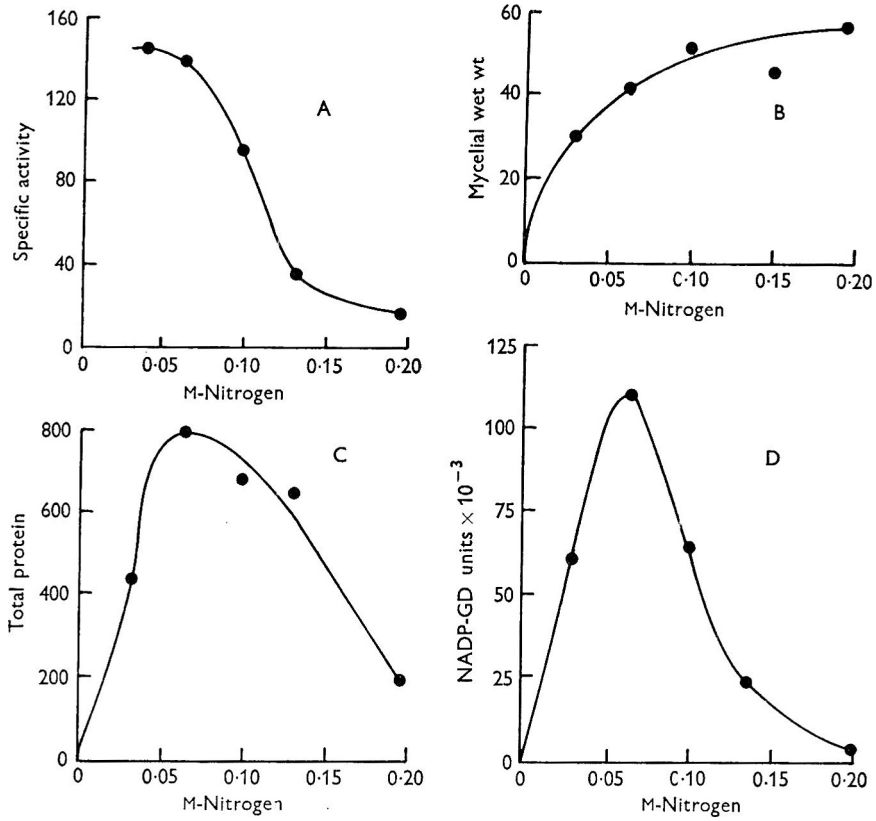

Fig. 1. The relationship between NH_4^+ concentration and mycelial growth of Neurospora, protein synthesis, NADP-GD synthesis, and NADP-GD-specific activity. Growth conditions: 1·6 l. medium F containing 2% (w/v) sucrose and 5 μg. biotin/l.; forced aeration at 33°; inoculum, a conidial suspension of strain STA 4; incubation period 48 hr. Protein determinations and NADP-GD assays made on non-heat treated extracts. NH_4^+ concentration expressed as molarity of nitrogen. A, NADP-GD specific activity expressed as units/mg. protein; B, mycelial weight expressed as g. wet weight; C, protein expressed as total mg.; D, NADP-GD expressed as total units $+10^{-3}$.

Table 2. *Effect of post-growth nitrogen starvation and reversal with* NH_4^+

Neurospora mycelium grown in 50 ml. medium N* containing 2% (w/v) sucrose. Mycelium incubated for 48 hr. at 33° on rotary shaker followed by treatment indicated below. Inoculum: filtered conidial suspension of strain STA 4. Assays on non-heat treated mycelial extracts.

Treatment	Growth period (days)	Total protein (mg.)	Specific activity (u./mg.)
None (control)	2	8·2	45
Nitrogen starved for 2 days	4	7·6	100
Nitrogen starved for 6 days	8	3·8	115
Nitrogen starved for 6 days, then supplemented with NH_4 tartrate,† harvested after 6 hr.	8·25	11·8	159

* Final pH of culture media 5·0 to 5·2. Medium N is 0·050 M with respect to nitrogen.

† Concentration brought to 0·13 M-nitrogen with ammonium tartrate.

significantly, when 6-day nitrogen-starved mycelium was supplemented with NH_4 tartrate to 0·13 M-nitrogen and allowed to grow for a further 6 hr., a nearly fourfold increase in total protein and an additional 50 % increase in specific activity was found.

Concurrent variation in concentration of ammonium ion and time of growth

As shown above, NADP-GD production was markedly altered by NH_4^+ concentration or post-growth nitrogen starvation. The effect of the two concentrations of NH_4^+ for two different periods of growth was investigated. Two flasks of medium F, one supplemented with 0·016 M-nitrogen and the other with 0·065 M-nitrogen (both as NH_4 tartrate), were inoculated with a very heavy conidial suspension (final concentration of 4×10^7 conidia/ml. culture medium). After incubation for 18 hr. one half of the mycelium from one flask of each concentration was harvested and the remainder harvested after incubation for 24 hr. At either concentration of nitrogen the specific activities were higher at 18 hr. than at 24 hr. (Table 3), but the differ-

Table 3. *Effect of various concentrations of* NH_4^+ *and growth period*

Neurospora mycelium grown in 2 l. medium F containing 2 % (w/v) sucrose + 5 μg. biotin/l. Aerated by bubbling at 33°. Inoculum 8×10^{10} conidia, strain STA 4. Data corrected for differences in volumes harvested. Assays on non-heat treated mycelial extracts.

Nitrogen (ammonium tartrate) (M)	Growth period (hr.)	Total protein (mg.)	NADP-GD specific activity (u./mg.)
0·065	18	458·0	216·0
0·016	18	365·0	222·0
0·065	24	380·0	181·0
0·016	24	297·0	139·0

Table 4. *Effect of time of harvest under conditions of early nitrogen starvation*

Neurospora mycelium grown in 1 l. medium F containing 2 % (w/v) sucrose + 5 μg. biotin/l. Aerated on shaker at 33°. Inoculum $1·4 \times 10^{10}$ conidia strain STA 4. No nitrogen supplied for 16 hr. Nitrogen supplemented to 0·065 M with ammonium tartrate.

Growth period after supplementation (hr.)	Total protein (mg.)	NADP-GD specific activity (u./mg.)
10	218	301
18	554	193

ence was more marked at the lower nitrogen concentration. Addition of optimal nitrogen (to 0·065 M) after 18 hr. growth was without effect. Inconsistencies exist between the data from the two different nitrogen starvation experiments. The mycelium which was nitrogen starved for 6 days showed a marked response in NADP-GD and protein synthesis within 6 hr. after nitrogen supplementation (Table 2). In contrast, mycelium grown for 18 hr. on limited nitrogen, followed by growth for 6 hr. on adequate nitrogen, showed no increase in NADP-GD or protein

synthesis. An experiment was therefore made in which nitrogen starvation preceded growth. Nitrogen-free medium F was inoculated with a heavy suspension of conidia, with reliance for conidial germination on endogenous nitrogen reserves. After incubation for 16 hr. the medium was supplemented with NH_4 tartrate to the optimal concentration (equiv. 0·065 M-nitrogen; see Fig. 1), the mycelium incubated for a further 10–18 hr. and harvested (Table 4). Microscopic examination of the inoculated media just before supplementation showed that most conidia had germinated but that mycelial growth was insignificant. When nitrogen was supplied and incubation was only for 10 hr., the specific activity was the highest obtained in any experiment. When incubation was for 18 hr., the total units of NADP-GD almost doubled and the total protein increased almost 2·5-fold. These data support the idea of early NADP-GD synthesis in relation to total protein synthesis and confirm the marked stimulatory effect of NH_4^+ on nitrogen-starved mycelia.

Effect of carbon source

Since ammonium ion has a marked regulatory effect on NADP-GD activity, variations in the other substrate α-ketoglutarate might also exert regulatory effects. Because of the instability of α-keto acids at the pH value of the culture medium used, no direct test seemed feasible. One indirect approach would be to attempt regulation of the concentration of tricarboxylic acid cycle intermediates by altering the concentration of carbohydrate. Different concentrations of sucrose showed maximal NADP-GD activity at 1 % (w/v), the lowest concentration used (Table 5). When the sucrose concentration was only 0·1 % there was very poor mycelial growth and low NADP-GD yields. Neurospora cannot use glutamate as sole carbon source, so that a direct test of end-product repression was not possible.

Table 5. *Effect of sucrose concentration under conditions of early nitrogen starvation*

Neurospora mycelium grown in 1 l. medium F containing 5 μg. biotin/l. Aerated on shaker at 33°. Inoculum $1·4 \times 10^{10}$ conidia strain STA4. No nitrogen supplied for 16 hr. Nitrogen supplemented to 0·065 M with ammonium tartrate. Incubation 18 hr. after supplementation.

Sucrose (%, w/v)	Total protein (mg.)	Specific activity (u./mg.)
1·0	454	274
2·0	554	193
5·0	512	150

Inhibitory effect of glutamate

Fincham (1954) and Sanwal & Lata (1962*a*, *b*) showed repression of NADP-GD by glutamate in the presence of NH_4^+. Similar experiments have now been made with four wild-type strains. In all cases glutamate in a molar ratio of 0·25 with respect to total nitrogen repressed NADP-GD formation. The repression observed was qualitatively but not quantitatively in agreement with the recent findings of Sanwal & Lata (1962*a*, *b*) in which glutamate was used in a molar ratio 0·50 with respect to total nitrogen.

DISCUSSION

During growth of Neurospora wild-type mycelia, total protein and NADP-GD synthesis was markedly influenced by the chemical form in which nitrogen was supplied. Of the nitrogen source investigated highest NADP-GD-specific activity and protein synthesis were consistently obtained when NH_4^+ was the nitrogen source. Since NADP-GD catalyses the reductive amination of α-keto acids in Neurospora (Fincham, 1950), correlation under these conditions between high protein synthesis and high NADP-GD production is not unexpected. However, glutamate, the product of the synthetic activity of the enzyme, is not a sufficient single source of nitrogen for high protein synthesis even when supplied at three times the nitrogen concentration of NH_4^+. At concentrations of NH_4^+ up to the optimal for protein synthesis, NADP-GD and protein synthesis increased in parallel, i.e. the specific activity remained constant. Above the optimal value NADP-GD-specific activity decreased sevenfold with a fourfold increase in NH_4^+, and with no effect on mycelial growth. A repressing effect of NH_4^+ or some compound in the nitrogen pool seems indicated.

Similarly, the depressing effect of glutamate alone, glutamate + NH_4^- or a mixture of amino acids, on specific activity support the contention that excess nitrogen in some form acts as a repressor. Sanwal & Lata (1962*b*) concluded that urea is the repressor of NADP-GD. However, their data differ from those reported here in that they found that replacement of urea with equivalent amounts of NH_4^+ (presumably 0·24 M with respect to nitrogen) had no significant effect on specific activity. Data reported here show a 90% decrease in specific activity when 0·195 M-nitrogen was used instead of 0·065 M-nitrogen (as NH_4 tartrate; see Fig. 1). Thus these data do not indicate urea as the repressor. Evidence reported by Sanwal & Lata (1962*b*) on the substitution of alanine, ornithine, or valine for the repressing effect of glutamate in the presence of NH_4^+ is conflicting. High alanine, ornithine, and valine transaminase activities have been reported in Neurospora in the presence of the respective specific substrates (Fincham & Boulter, 1956); yet Sanwal & Lata (1962*b*) found that alanine and ornithine but not valine substituted in reproducing the glutamate depression effect. An understanding of the mechanism of NH_4^+ repression awaits the application of short-time induction experiments in which the environment remains relatively constant during the experiment, such as those with washed mycelia recently reported in studies with nitrate reductase regulation in Neurospora (Kinsky, 1961).

Ammonium ion *per se* may actually act to de-repress NADP-GD production. Specific activity is low in conidia and increases nearly eightfold within the first 24 hr. of incubation (nitrogen supplied as a mixture of NH_4^+ and NO_3^- (Sanwal & Lata, 1962*a*). Extreme restriction of NH_4^+ by growth of mycelium in presence of glycine, serine and lysine results in poor growth but high specific NADP-GD activity. When severely nitrogen-starved mycelium is supplemented with NH_4^+, a rapid reversal of soluble protein and NADP-GD catabolism is observed, as well as an increase in specific activity. Conidia germinated in nitrogen-free media and then supplemented with NH_4^+ yielded mycelium with the highest NADP-GD specific activity observed under any experimental conditions, and decreasing with time. Because of the unique role of this enzyme in the incorporation of NH_4^+ into

α-amino nitrogen, complicated by regeneration of α-aminoglutarate via transaminations, the regulatory mechanism of induction and repression of NADP-GD may be complex and elusive.

To obtain maximal total NADP-GD synthesis, the concentration of NH_4^+ and nitrogen starvation appear to be significant. The highest NADP-GD yields accompanied by high specific activity were obtained under conditions in which nitrogen-starved mycelium, germinating on endogenous reserves, was harvested 10 hr. after supplementation with NH_4^+. Under these conditions more than 1 % of the soluble protein in heat-treated fractions was found to be NADP-GD. While NADP-GD synthesis continued after 10 hr. the total protein synthesis occurred more rapidly, resulting in a lower specific activity.

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An Electron-Microscope Study of Germination of Conidia of *Botrytis cinerea*

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SUMMARY

Conidia of *Botrytis cinerea* have a 2-layered cell wall, a thin electron-dense outer layer and a thicker electron-transparent inner one. The cell contents comprise typical nuclei, mitochondria and endoplasm with a surrounding plasmalemma and a sparse endoplasmic reticulum. There are a few small peripheral vacuoles. On germination the outer spore wall ruptures and the emergent germ tube is surrounded by the elastic inner one and what appears to be a sheath of mucilage. A cross wall with a simple central pore is laid down at the base of the germ tube at a very early stage. As the germ tube elongates the contents of the spore flow into it, causing a large vacuole to form in the centre of the original spore. The numbers of nuclei and mitochondria suggest that divisions of these organelles take place during the early stages of germination. The mucilaginous sheath surrounding the germ tube appears to condense to give a thin electron-dense outer zone to the wall at the base of the germ tube.

INTRODUCTION

Hawker & Abbott (1963) showed that the mature sporangiospores of species of Rhizopus have a relatively thick single-layered cell wall of structure unlike that of the hyphal wall; after soaking in water for 3 hr. a thin inner wall can be seen lining the original cell wall. This inner wall consists of tangentially arranged elements and is similar to the walls of young vegetative hyphae. On germination of the spore the original spore wall breaks and the germ tube emerges surrounded by the elastic newly formed inner wall. If the conidium, the typical asexual spore of the Ascomycetes and Fungi Imperfecti, has evolved from the more primitive multispored sporangium by the reduction of the number of spores to one, one would expect this spore to have initially 2-layered walls. Accordingly conidia of *Botrytis cinerea* Fr. were fixed, embedded in araldite, sectioned and examined under the electron microscope. The techniques were similar to those described by Hawker & Abbott (1963) except that the sections were stained with sodium plumbite to enhance contrast and most of them were examined with a Siemens Elmiskop I electron microscope instead of the Phillips E.M. 200 electron microscope used in previous studies.

RESULTS

Fine structure of conidium

Spore wall. Conidia taken from a mature culture of *Botrytis cinerea* and fixed immediately (Pl. 1, fig. 1) have relatively thick walls (0·5 μ) consisting of two ill-defined layers. The outer thinner layer (OW) is electron-dense; its outer surface is

rough and fluffy but shows no regular ornamentation of the type seen in sporangiospores of Rhizopus; its inner edge is not sharply demarcated. The thick inner wall (IW) is electron-transparent; no structure can be demonstrated by the methods used except that dark round elements are present in the zone immediately adjoining the outer wall.

Cell contents. (i) A distinct plasmalemma (P , Pl. 1, fig. 1) is present, closely adpressed to the cell wall. Lomasomes (Moore & McAlear, 1961) are common in the plasmalemmas of longer germ tubes (Lo, Pl. 2, fig. 7). (ii) The endoplasm consists of a homogeneous matrix in which scattered membranes (ER) are present. Cisternae or vesicles are rare. A few very small peripheral stellate vacuoles (SV) are present in some spores. (iii) The nuclei (N) are few, relatively small (2–2·5 μ), and surrounded by the typical 3-layered membrane perforated by pores (NP). (iv) Numerous small mitochondria (M) are present. These are of varied size and shape, commonly oval, rod-shaped (M, Pl. 1, fig. 1) or U-shaped as seen in section (M_1 and M_2 in Pl. 2, fig. 8). Occasionally (M_3 in Pl. 2, fig. 7) a convoluted mitochondrion occurs resembling those demonstrated in 'dormant' spores of Rhizopus by Hawker & Abbott (1963).

Stages in germination

Germination of conidia took place after soaking for 6 hr. in water at 20°. Light-microscope studies showed that the first visible stage in germination was the swelling of the spores through intake of water and the germ tube then emerged. In the absence of a complete series of ultra-thin sections through a number of spores it was not possible to note increase in spore size in electron micrographs. Early stages in the emergence of the germ tube may, however, be seen. Although it was not possible to be certain that a section had gone through the tip of the germ tube, criteria such as the size of the vacuole developing in the parent spore and the condition of the germ tube wall permit an estimate of the relative ages of the photographed germ tubes.

Plate 1, figs. 2 and 3, and Pl. 2, figs. 4 and 5, give a series of stages in germination; figs. 2 and 3 show very early stages. The outer electron-dense spore wall has ruptured in a manner reminiscent of the spore wall of Rhizopus and the emerging germ tube is surrounded by the extending thicker inner spore wall. Where this was in contact with the surrounding medium a diffuse deposit is seen. This probably represents the mucilaginous layer, known to be present, which serves to stick the Botrytis germ tube to a leaf or other firm surface, before the formation of an appressorium (Blackman & Welsford, 1916). No large central vacuole has as yet developed in the parent spore, indicating that very little of the original spore contents has passed into the young germ tube. Even at this early stage a cross wall has formed at the base of the germ tube. In Pl. 2, figs. 5–7, serial sections show that this basal septum has a central pore through which cytoplasm and organelles can pass. Plate 2, fig. 4, shows a later stage as indicated by the large central vacuole in the parent spore (shown also in sections through germinated spores; Pl. 2, fig. 8) and the presence of nuclei and numerous mitochondria in the germ tube, which itself contains no vacuoles. The number of nuclei seen (Pl. 2, figs. 4, 5) suggests that one or more nuclear divisions have already taken place. Plate 2, fig. 5, shows a still later stage: a vacuole has developed near the base of the germ tube and the wall enclosing this basal part has become more sharply defined with the development of a thin electron-

dense outer layer which appears to be the result of condensation of the diffuse mucilaginous sheath. Blackman & Welsford (1916) figured this as a continuation of the spore wall, but the electron micrographs show that this is not so. The pore in the basal septum (Pl. 2, fig. 6) is clearly visible, with a mitochondrion in process of passing into the germ tube.

DISCUSSION

The evolution of the conidium is a matter of considerable interest and has been the subject of much speculation. Members of the Mucorales can be placed in a series which shows a decrease in numbers of spores in the sporangium from the multi-spored sporangium of Mucor, Rhizopus and related forms to species such as Chaeto-cladium or Cunninghamella in which the 'conidia' are assumed to be single-spored sporangia. The conidium is the prevailing asexual spore type in the remaining groups of the Zygomycetes (Entomophthorales and Zoopagales). In the Oomycetes there is an increasing tendency to germination of the zoosporangium as a single spore by means of a germ tube under relatively dry conditions, culminating in Peronospora which does not form zoospores under any circumstances. If the conidium of the Ascomycetes and Fungi Imperfecti has evolved from the Zygo-mycete type of sporangium one would expect it to have a double wall laid down at the time of formation of the spore initial. If, however, it has evolved from the Oomycete type of zoosporangium, in which the zoospores are without walls, one would expect the conidium wall to be initially of only one layer. The evidence given in this paper supports the view that the Zygomycetes and Ascomycetes (including the majority of the Fungi Imperfecti) have evolved from a common ancestor. The outer wall of the Botrytis conidium would then be homologous with the sporangial wall of Rhizopus and the inner wall with the initial spore wall of the sporangiospore of Rhizopus. A difference arises in the extension of the inner wall of the conidium of Botrytis to surround the developing germ tube, whereas the spore wall of Rhizopus ruptures and the emergent germ tube is surrounded by a newly formed inner wall which is produced rapidly when the sporangiospore is soaked in water or nutrient liquid. No membrane homologous with this was seen in Botrytis. Such an addi-tional membrane is, however, unnecessary in a spore which has a 2-layered wall from the start, and may well have been lost during evolution. Alternatively the single-layered wall of the Rhizopus spore may formerly have extended round the germ tube and when this wall became thickened and inelastic the new inner wall may have evolved. The 2-layered wall gives an enhanced chance of resistance to desiccation and other unfavourable environmental conditions. Its possession may justifiably be considered to be a more advanced condition than the possession of a wall of only one layer.

The presence of a septum at the base of the germ tube from an early stage is of interest. Unfortunately, no section of the large number examined showed early stages in the formation of this septum. This is not surprising in view of the known rapidity of septum formation in other fungi (Bracker & Butler, 1963). The pore in the basal septum is of simple structure and would allow practically unimpeded passage of cytoplasm and organelles from the spore into the germ tube. No complex protecting structures, such as those shown by Moore & McAlear (1962) in certain Ascomycetes or the more complex ones shown by the same authors and by Bracker

& Butler (1963) in certain Basidiomycetes, were observed. Portions of the endoplasmic reticulum were, however, usually aligned parallel to the septum (Pl. 1, figs. 2, 3). Pores of mature hyphae of Botrytis were not observed and no further septa were present in such young germ tubes as those illustrated. More complex structures may well develop in this species with ageing of the septa. No such basal septum was present in the germ tubes of Rhizopus spores studied by Hawker & Abbott (1963)

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EXPLANATION OF PLATES

PLATE 1. *Botrytis cinerea*

Fig. 1. Approximately transverse section of an ungerminated conidium showing relatively thin, electron-dense, fluffy outer wall (OW); relatively thicker inner wall (IW) showing scattered dark, round elements in zone adjoining outer wall; plasmalemma (P); nucleus (N) with at least one nuclear pore (NP); mitochondria (M) with entire outlines and clearly defined cristae; a few peripheral stellate vacuoles (SV); and sparse endoplasmic reticulum (ER).

Fig. 2. Approximately median transverse section of a conidium at an early stage of germination. The germ tube (GT) enclosed by the inner wall (IW) has emerged through the outer wall (OW) at X–X (note broken edge of outer wall); on contact with the surrounding medium a layer of mucilage (Muc) is deposited. Already a basal septum (S) has been laid down, though a septal pore is not visible in this section. Other lettering as in Fig. 1.

Fig. 3. Slightly oblique section of germinating conidium at a later stage than Fig. 2. Lettering as in Fig. 2.

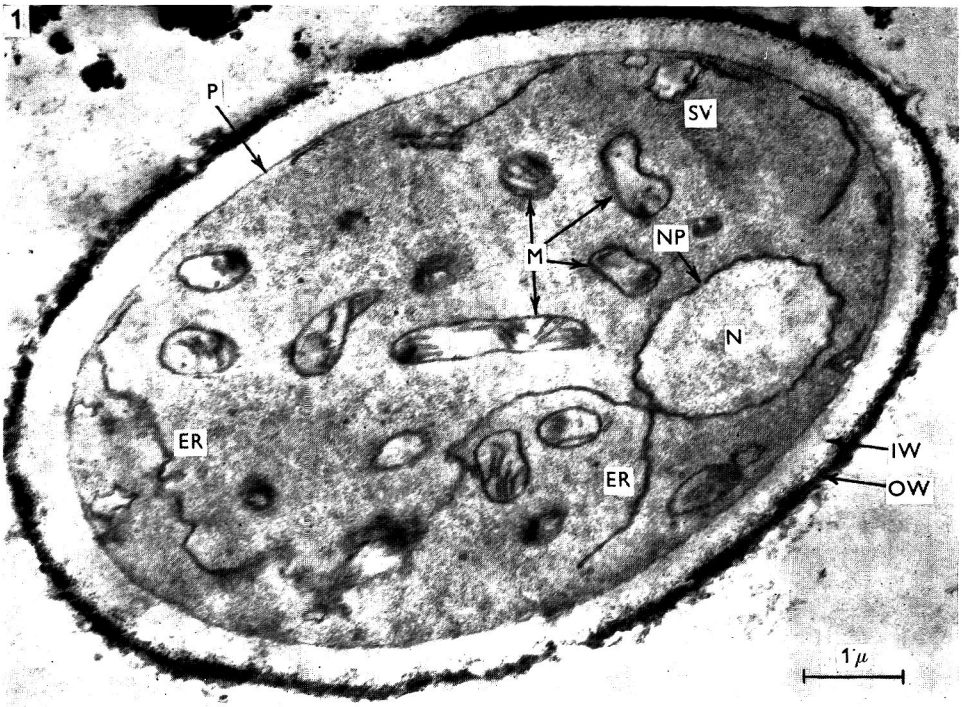
PLATE 2. *Botrytis cinerea*

Fig. 4. Longitudinal section of a germinating conidium at a somewhat later stage than Fig. 3. In the original spore a large central vacuole (V) has formed due to passage of endoplasm into the germ tube which now contains a number of nuclei (N), mitochondria (M) and a small vacuole (V). The increasing number of nuclei (at least four in this specimen) suggests that several nuclear divisions have already taken place. Many more mitochondria are now present within the conidium.

Fig. 5. Germinated conidia at a still later stage. A very large vacuole is now present in the old spore, and the mucilaginous sheath (Muc) appears to have condensed to give a thin electron-dense layer at the base of the germ tube. Five or more nuclei are present in the spore at the upper left of the figure.

Figs. 6, 7. Serial sections of the basal septum of the spore in the lower left of Fig. 5. A septal pore (SP) is clearly visible, with a mitochondrion in the act of passing through. Lomasomes (Lo) are found in the germ tube but have not been seen within the conidium. The contorted mitochondria (M_3 in Fig. 7) resemble those demonstrated in dormant spores of Rhizopus.

Fig. 8. Approximately transverse section of a conidium at a late stage of germination, as shown by the large vacuole (V). Mitochondria M_1 and M_2 have an interesting shape which may indicate a stage in their division.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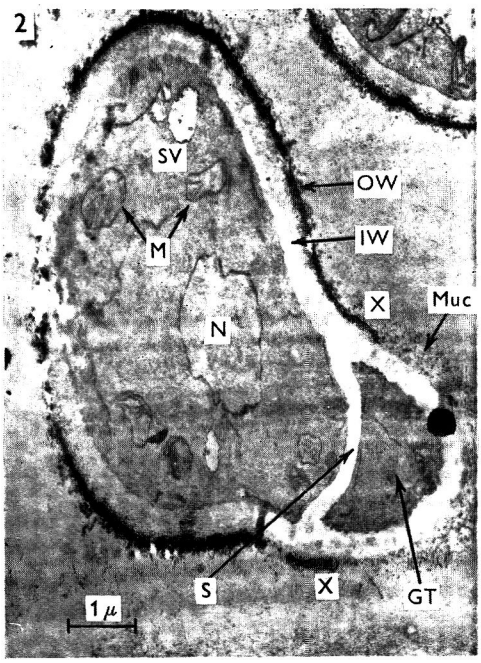
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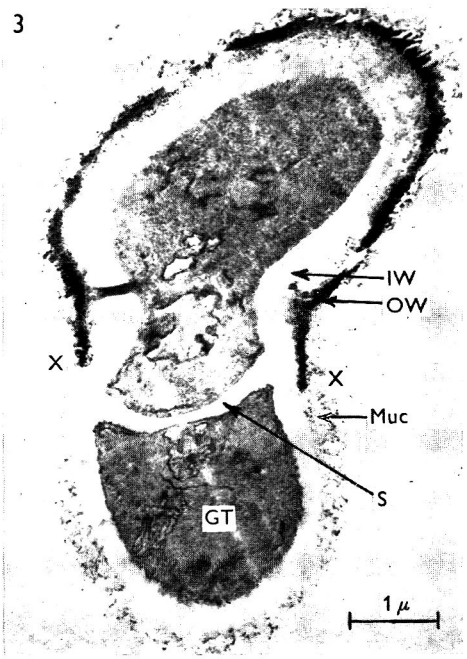
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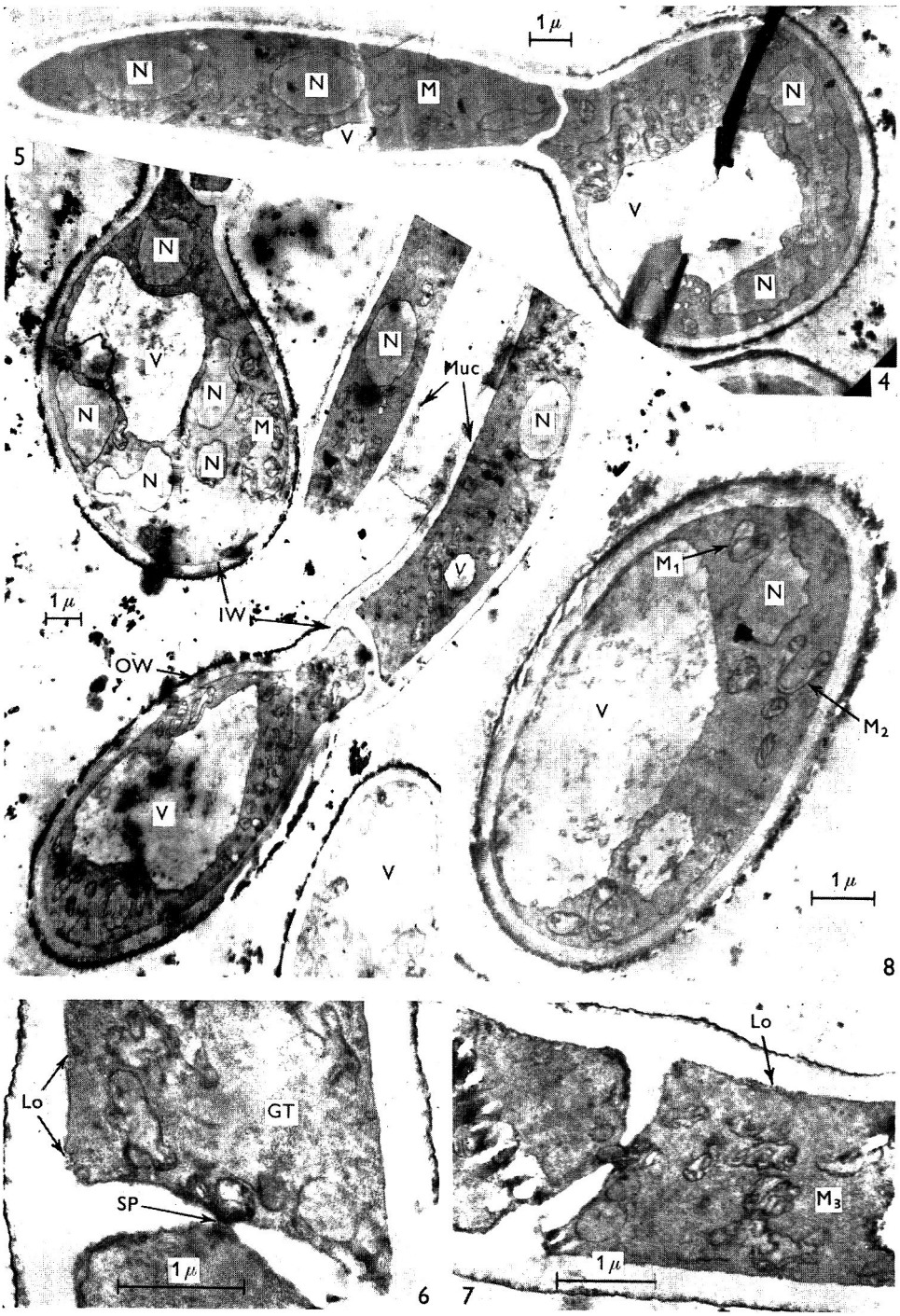
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Size of Inoculum and Carbon Metabolism in some Aspergillus Species

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SUMMARY

The effect of size of inoculum on growth and carbon metabolism of *Aspergillus oryzae* and several other Aspergillus species was studied. During most of the development a culture from a small inoculum, as compared to a large-inoculum culture, had a smaller specific rate of respiration, formed more ethanol and acids per unit weight of mycelium, and metabolized more carbohydrate to produce a given amount of growth. It seems that this inefficient utilization of carbon source is due not only to the production of a smaller amount of energy but also to an inefficient utilization of the released energy. The higher degree of inefficiency appears to be the result of the aging process which a small-inoculum culture undergoes during its development to the stage of growth of a young large-inoculum culture; the latter also shows a decrease in efficiency during cultivation.

INTRODUCTION

In earlier publications it was reported that certain growth characteristics of cultures of *Aspergillus oryzae* were influenced by the size of the inoculum (Meyrath, 1962*a*, 1963). Conidia were then mainly used as inoculum; a large and a small inoculum comprised usually 4×10^7 and 4×10^3 conidia/100 ml. substrate, respectively (corresponding to dry weights of organism of about 0·8 and $0{\cdot}8 \times 10^{-4}$ mg.). Alternatively, filamentous mycelium was used as inoculum, the corresponding amounts being about 10 and 10^{-3} mg. dry wt. mycelium/100 ml. culture. In many substrates rate of growth and maximum yield decreased when a small inoculum was used. Under the particular conditions of using substrates either poor in trace elements or rich in carbohydrate the effects of inoculum size were very marked and were even more pronounced when both conditions obtained simultaneously. The effect on maximum yield of organism was always more pronounced in submerged cultures with vigorous agitation and aeration than in stationary or shaken cultures. It may also be mentioned here that the pronounced effects obtained in trace-element-poor substrates were not due to a carry-over of trace elements contained in large inocula.

To understand the phenomena observed with different sizes of inocula, the influence of size of inoculum on characters other than mycelium formation was examined. The present work deals mainly with the effect of size of inoculum on the metabolism of carbon sources.

METHODS

Organism and inoculum. The *Aspergillus oryzae* strain used for most of the work described was that used previously (Meyrath, 1962*a*, 1963). This organism and the strain of *Aspergillus flavus* used were stock strains in the Department of Agricultural Bacteriology and Fermentation, Swiss Federal Institute of Technology, Zürich. The other Aspergillus strains mentioned were obtained from the Centraalbureau voor Schimmelcultures, Baarn, Netherlands. Conidia were used as inoculum, and were at first produced on malt wort agar slopes by incubation at 25° for 7–14 days. Later it was found that sporulation was heavier and more reliable under the same conditions when the substrate described below was used. In neither case did size of inoculum influence the extent of formation of conidia. A suspension of conidia in sterile distilled water was filtered through sterile absorbent cotton wool. The conidia were washed three times by centrifugation, counted in a haemocytometer and diluted as required. Their germinative ability was determined on malt wort agar plates after incubation for 3 days at 25°. This value has remained consistently at about 50 % over several years of testing.

Table 1. *Composition of the substrates*

All substrates contained (g./l.): carbohydrate (starch 10; if otherwise, kind and concentration are specified in the text); $(NH_4)_2SO_4$, 1; $MgSO_4.7H_2O$, 0·3. The other components are as indicated below.

	A	A1	A3	A4	A5
			g./l.		
Na_2HPO_4	4·73	4·73	9·46	4·73	9·46
KH_2PO_4	4·54	4·54	9·08	4·54	9·08
			mg./l.		
$FeCl_3.6H_2O$	15·0	15·0	15·0	15·0	2·5
$CuSO_4.5H_2O$	0·5	—	—	0·05	0·2
$MnSO_4.4H_2O$	0·5	—	—	0·05	0·2
$ZnSO_4.7H_2O$	5·0	—	—	0·5	Varied
$CaCl_2.6H_2O$	10·0	—	—	1·0	—

Substrates. The substrates used were in part those described elsewhere (Meyrath, 1963). Their composition is given in Table 1.

The sporulation substrate contained (g./l.): starch, 40; $(NH_4)_2SO_4$, 8; $MgSO_4.7H_2O$, 0·3; Na_2HPO_4, 35·5; KH_2PO_4, 34; $FeCl_3.6H_2O$, 0·003.

All chemicals were of Analar or *Pro analysi* (Merck) quality with the exception of the sucrose which was a commercial product.

The pH value of the media described is approximately 6·8; they undergo no significant change of pH value during sterilization.

Starch when used was poured into a boiling solution of the remaining constituents; the boiling was maintained for a few minutes, then the cooled solution made up to volume. When glucose was used, the mineral solution was autoclaved separately. When starch or sucrose was used, minerals and carbohydrate were sterilized together, starch medium by autoclaving, sucrose medium by tyndallization. Tyndallization of small amounts of substrate was by steaming for 30 min. on 3 consecutive days.

Procedure. Submerged cultures were grown in 5 l. quantities in 6 l. triple neck flasks, fitted with a Vibro-Mix stirrer (AG. für Chemie-Apparatebau, Männedorf, Zürich, Switzerland) which agitated the substrate by a vibrating stirrer; the degree of agitation could be adjusted by the amplitude of the vibrations and the size of the stirrer. The culture was aerated at a flow rate of about 500 ml./min. Homogeneous growth of dispersed mycelium was obtained. The substrate received an inoculum of 2×10^7 conidia/100 ml. medium (large-inoculum experiments), or 4×10^3 conidia/100 ml. (small-inoculum experiments), the inoculum being 50 ml. in each case. Stationary cultures were grown in 50 ml. or 100 ml. conical flasks containing 20 or 25 ml. medium, respectively. Each flask was inoculated with 1 ml. of suspension containing 10^7 conidia (large-inoculum experiments) or 10^3 conidia (small-inoculum experiments).

The temperature of incubation was 25° in all experiments.

From submerged cultures, with filamentous mycelium formation, samples of 10–500 ml. were taken so that the mycelium dry weight of each was not less than 5 mg.; estimations were done in duplicate or triplicate. With stationary cultures, duplicate or triplicate sample flasks were taken. The mycelium in both cases was collected on nylon filters in Büchner funnels. After repeated alternate washing with water and pressing with a rubber bung, pellets were made, dried at 104° overnight, weighed and the weights at each stage calculated on a basis of mg. dry wt. mycelium/100 ml. culture.

Oxygen uptake was measured manometrically or volumetrically, and output of carbon dioxide manometrically. Homogeneous filamentous mycelium from Vibro-Mix cultures was used for these experiments; mycelium washed and pressed as described, but undried, was suspended in water or phosphate buffer (Sörensen, M/15, pH 6·8). The gas exchanges of unwashed cell material were also studied with mycelium taken directly from the culture or from material concentrated on a nylon filter and suspended in culture filtrate. To ensure a suitable working range, the mycelium wet weight of duplicate samples was determined and dilutions of the test samples made. The manometric experiments were done with about 2 to 3 mg. dry wt. mycelium/flask. With the volumetric technique between 20 and 30 mg. dry wt. mycelium/flask were used; the mycelium was tested within about 3 hr. from the time of sampling.

Flasks (13–23 ml.) for manometric measurement received 2 ml. mycelial suspension in the main compartment and 0·5 ml. glucose solution (10 mg./ml.) or buffer in the side arm. For the estimation of oxygen uptake, 0·2 ml. of 20 %KOH was placed in the centre well the inner rim of which was coated with lanolin to prevent creeping of the alkali. The manometers were shaken at 103 cyc./min.; amplitude 4·2 cm.; in other respects the procedure described by Umbreit, Burris & Stauffer (1949) was followed. Flasks for volumetric measurement of about 100 ml. capacity fitted with a rotatable side arm were attached to 10 ml. burettes by rubber bungs; they received 20 ml. mycelial suspension in the main compartment, 1 ml. glucose solution (20 mg./ml.) or buffer in the side arm and 1 ml. 20 % KOH in the centre well (treated as for the manometric flasks); for further details see Nilsson (1941). All gas exchange experiments were done in duplicate at 25°. Carbon dioxide was estimated by the direct method of Warburg. With buffer or substrate as suspending medium for the mycelium, the pH value was determined and the solubility

of carbon dioxide calculated according to the formula $\alpha' = 0{\cdot}759$ [antilog $(pH - 6{\cdot}34) + 1$], α' indicating the solubility of carbon dioxide at the given pH.

The gas exchange reactions were usually recorded at 15 min. intervals over 3 hr. In all cases zero order reactions were obtained, with hardly any induction phase.

Sugar was estimated after Stiles, Peterson & Fred (1926); before assay, starch was hydrolysed by heating in a boiling water bath for 1 hr. in N-HCl and the mixture neutralized with NaOH.

Ethanol was estimated after Maxon & Johnson (1953). Although not specific, this method was used because ethanol appears to be the only volatile oxidizable compound present in measurable amount in cultures of *Aspergillus oryzae* (Tamiya, 1942). pH values were determined by a calomel/glass electrode system.

RESULTS

Oxygen uptake and carbon dioxide output

It has been reported that the specific rate of oxygen uptake (oxygen uptake/unit weight cell material/time) by filamentous fungi, actinomycetes and bacteria decreases with increasing age of the culture (Tamiya, 1942; Rolinson, 1952; Somm, 1953; Di Marco, 1956; Temperli, 1956; Müller, 1957). So far, however, it does not appear to have been shown that this holds during the phase of constant multiplication rate, i.e. the exponential phase, where a regular increase of cellular material is expected to take place. When a submerged culture of *Aspergillus oryzae* suitably provided with nutrients is kept under a sufficiently vigorous degree of aeration and agitation, it is possible to establish exponential growth over a wide range of the growth curve (Meyrath, 1953). When cultures from large inocula are examined under conditions of unrestricted growth, young cultures (up to 32 hr.) show a high specific rate of oxygen uptake (Q_{O_2} = 100–220 μl. O_2/mg. dry wt. mycelium/hr.), whereas older cultures are characterized by a much smaller value (30–56 μl. O_2/mg. dry wt. mycelium/hr.). One set of results is shown in Fig. 1, where the growth curves obtained from large and small inocula are represented. In the semi-logarithmic plot the difference in maximum yield of cell material between large- and small-inoculum cultures appeared to be small, but was in fact appreciable. The large-inoculum culture produced 430 mg. dry wt. mycelium/100 ml. medium and the small-inoculum culture 260 mg. dry wt. mycelium/100 ml. medium. Table 2 is a compilation of data on oxygen uptake from experiments under various conditions. When small-inoculum cultures were grown under the same conditions, mycelium was produced which gave much lower rates of specific oxygen uptake at all stages of measurable growth. The results of one set of experiments in the phase of exponential growth is shown in Fig. 1; further data obtained under other conditions revealed a similar phenomenon. One point to be stressed is that readings of oxygen uptake for large- and small-inoculum cultures were taken at equally low values of mycelium content.

The results suggest that mycelium from small-inoculum cultures at all stages of measurable growth behaved like mycelium from large-inoculum cultures at late stages of growth. It appears that small-inoculum cultures underwent an aging process (leading to a diminished rate of oxygen uptake) during the prolonged time they took to reach the same content of mycelium as in large-inoculum cultures at

early stages of growth. In these particular substrates, with submerged cultivation, the rate of multiplication remained constant for large-inoculum and small-inoculum cultures (Fig. 1; see also Meyrath, 1963) although the specific rate of oxygen uptake decreased. This means that in advanced stages of growth less oxygen was required to form a given weight of mycelium. If the amount of energy required for synthesis of unit weight of mycelium was constant during exponential growth, and if a given amount of oxygen taken up released a proportional amount of energy for cellular synthesis, then it might be assumed that in advanced stages of growth the required energy is made up by anaerobic processes. This view was expressed in connexion with anaerobic spoilage of fruit juices by moulds (Meyrath, 1962*b*). The result of this anaerobic metabolism is an inefficient utilization of carbon source. Since small-inoculum cultures are characterized by a low specific rate of oxygen uptake during

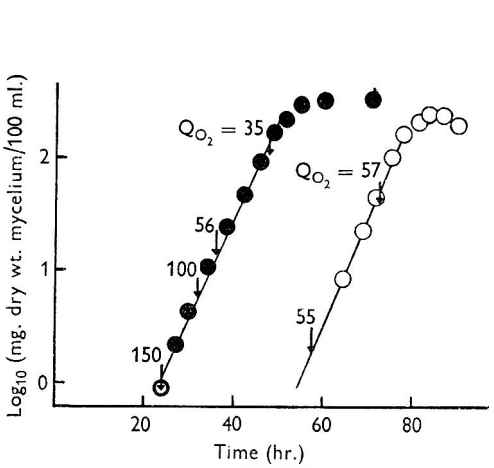


Fig. 1

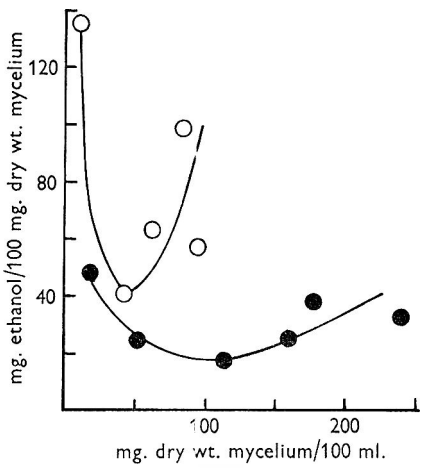


Fig. 2

Fig. 1. The accumulation of mycelium of *Aspergillus oryzae* and the specific oxygen uptake at different stages of growth, from large (●—●) and small (○—○) inocula.

Fig. 2. Specific ethanol production of *Aspergillus oryzae* in stationary cultures with substrate A 3, at various stages of growth using large (●—●) and small (○—○) inocula.

Table 2. *Specific rate of oxygen uptake and respiratory quotient (RQ) of mycelium from submerged large-inoculum cultures of* Aspergillus oryzae *at various stages of growth.*

Group of experiments	Stage of growth (mg. dwm.*/ 100 ml.)	Age of culture (hr.)	Substrate for mycelium production	Medium for suspension of mycelium	Rate of oxygen uptake (μl./mg. dwm.*/ hr.)	*RQ*
I	1·9	24	A	Culture	115	—
	22·6	36		filtrate + glucose	78	—
II	8·7	22	A4	Culture	155	1·11
	16·6	25		filtrate + glucose	190	1·03
	229·2	48			30	1·39
	310·8	96			30	1·03
	52·0	191			30	1·03

* dwm., dry weight mycelium.

measurable growth, they may be expected to utilize the carbon source less efficiently than large-inoculum cultures; the latter are characterized by a high specific rate of oxygen uptake in early measurable growth, an appreciable part of total growth in this instance.

That anaerobic dissimilation (anoxybiontic fermentation, according to Bernhauer) may take place to a larger extent in older cultures is indicated by the increase of the respiratory quotient (*RQ*) at some stages of growth (Table 2). Further evidence of this phenomenon is given below, together with the role which it plays when the size of the inoculum is varied.

Efficiency of utilization of the carbon source

Ethanol is a common fermentation product of *Aspergillus oryzae* (Tamiya, 1942) and its accumulation is necessarily accompanied by evolution of carbon dioxide without uptake of oxygen, leading to a higher respiratory quotient as shown previously. In the present work ethanol was estimated in stationary cultures, using

Table 3. *Influence of various concentrations of zinc on the ratio dry weight of mycelium from large inoculum: dry weight of mycelium from small inoculum at three different stages of growth of* Aspergillus oryzae

Time of cultivation (days)	Concentration of zinc (μg./100 ml.)					
	0	0·3	1	3	10	30
7–8	4·82	4·48	3·07	2·63	1·88	1·78
10–11	2·76	2·85	2·2	1·50	1·33	1·41
22–23	1·65	1·69	1·33	1·19	1·25	1·22

substrates A 3 (no trace elements added except Fe) and A 5 (different amounts of Zn and other trace elements added). Figure 2 shows that the ratio by weight of ethanol to mycelium (specific ethanol production), determined at various stages of the linear phase of growth, was always higher when a small inoculum was used. The growth curves corresponding to this experiment showed that a much smaller growth rate in the linear phase was evident when the inoculum was small. Even in the presence of trace elements (substrate A 5) the specific ethanol production was always higher in small-inoculum cultures than in large-inoculum cultures over a growth range of about 45–375 mg. dry wt. mycelium/100 ml. (Fig. 3). Beyond this range, when 3 or 30 μg. Zn were added/100 ml., about equal or slightly smaller specific amounts of ethanol were formed in the small-inoculum culture. In this substrate ethanol formation did not increase in late stages of growth contrary to the results obtained with substrate A 3.

The general effect of zinc, as well as its particular effect on ethanol production, may be mentioned here. It has been reported that trace elements counteract the effects of size of inoculum (Meyrath, 1962*a*, 1963). It can be seen from Table 3 that among the trace elements used in these and other experiments, zinc (1–30 μg./100 ml.) was particularly important in diminishing the effects of inoculum size. The ratio of dry wt. mycelium from large-inoculum to that from small-inoculum culture (dwm. large/dwm. small), determined at various stages of growth, decreased steadily with increasing concentration of zinc. The decrease of this ratio was due to the fact that zinc stimulated the growth of small-inoculum cultures more strongly

than that of large-inoculum cultures (see Fig. 4). Addition of zinc not only decreased the ratio dwm. large/dwm. small, but also suppressed specific ethanol production over the major part of the culture development (Fig. 3).

Although ethanol appears to be the major fermentation product of *Aspergillus oryzae* under the present conditions of testing, there was some formation of organic acids when the carbohydrate concentration was higher than 10 g./l. (at 1 g. ammonium sulphate/l.). *Aspergillus oryzae* and five other strains of Aspergillus were examined to discover to what extent this property was dependent on inoculum size.

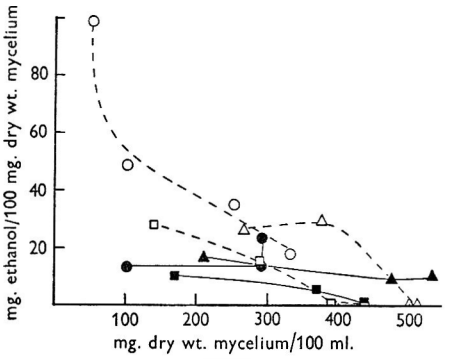


Fig. 3

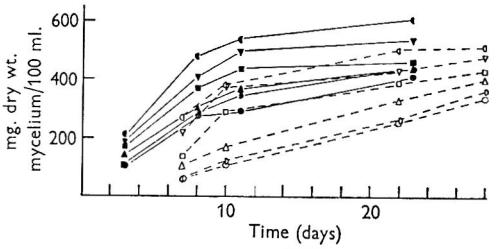


Fig. 4

Fig. 3. Specific ethanol production by *Aspergillus oryzae* in stationary cultures with substrate A5 at various stages of growth.

μg. Zn/100 ml.	large inoculum	small inoculum
0	●—●	○—○
3	■—■	□—□
30	▲—▲	△—△

Fig. 4. Growth of *Aspergillus oryzae* in stationary cultures with substrate A5.

μg. Zn/100 ml.	large inoculum	small inoculum
0	●—●	○—○
0·3	◗—◗	◗—◗
1	▲—▲	△—△
3	■—■	□—□
10	▼—▼	▽—▽
30	◖—◖	◖—◖

Figure 5 shows that in substrate A 1 (80 g. sucrose/l.) production of organic acids at the various stages of growth was higher in small-inoculum cultures. All strains showed the typical effects of inoculum size, i.e. decreased rate of growth in the linear phase and decreased maximum yield of mycelium when small inocula were used (Fig. 6). *Aspergillus oryzae* reacted more strongly to changes in inoculum size than did the other Aspergillus species examined. Since the production of organic acids is a low-energy-yielding process, the same conclusion is reached as with the studies on production of ethanol and carbon dioxide, and on oxygen uptake, namely, a small-inoculum culture is less efficient than a large-inoculum culture in its use of the available carbohydrate as energy source. This comparative inefficiency (with increased formation of fermentation products) should have its counterpart in the disappearance of the carbon source supplied. Figure 7 shows that a small-inoculum culture used more starch to form a given amount of mycelium at all stages of growth

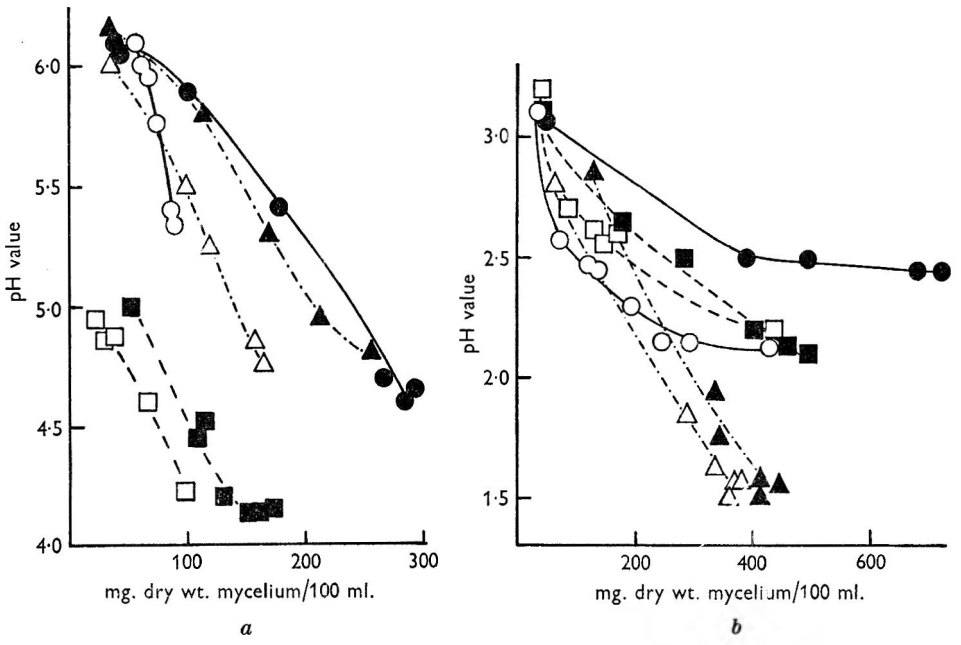
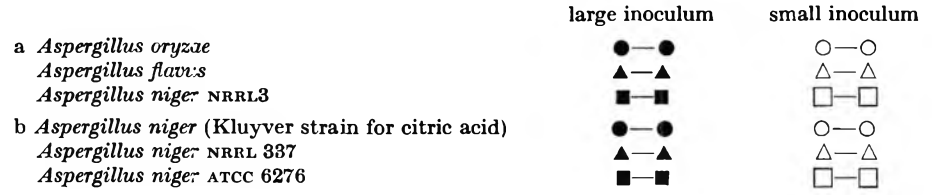


Fig. 5. Production of acids (measured by decrease in pH value) by several *Aspergillus* strains at various stages of growth.



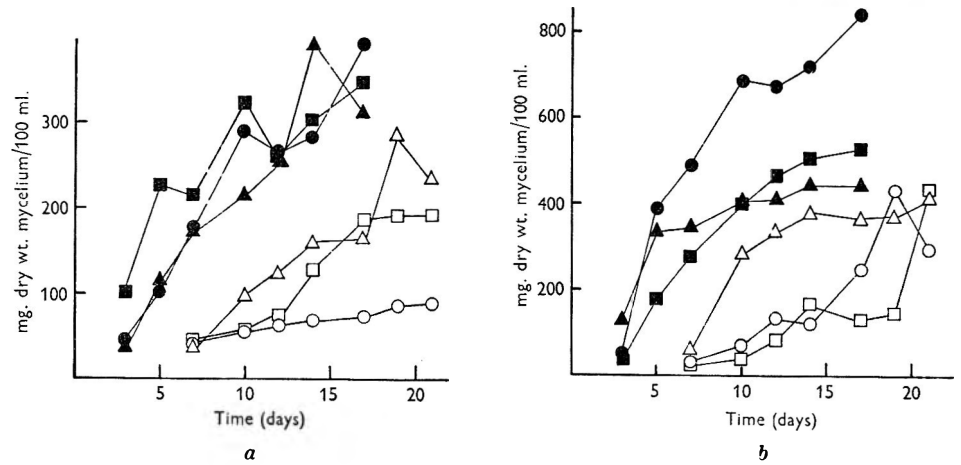


Fig. 6. Growth of various Aspergillus strainsi n stationary cultures using large and small inocula (symbols as for Fig. 5).

in substrate A 4. A similar phenomenon was observed in substrate A. In both cases all the starch was metabolized at the point where maximum yield of mycelium was attained. The result of this is that large-inoculum cultures give a higher maximum yield of mycelium than small-inoculum cultures.

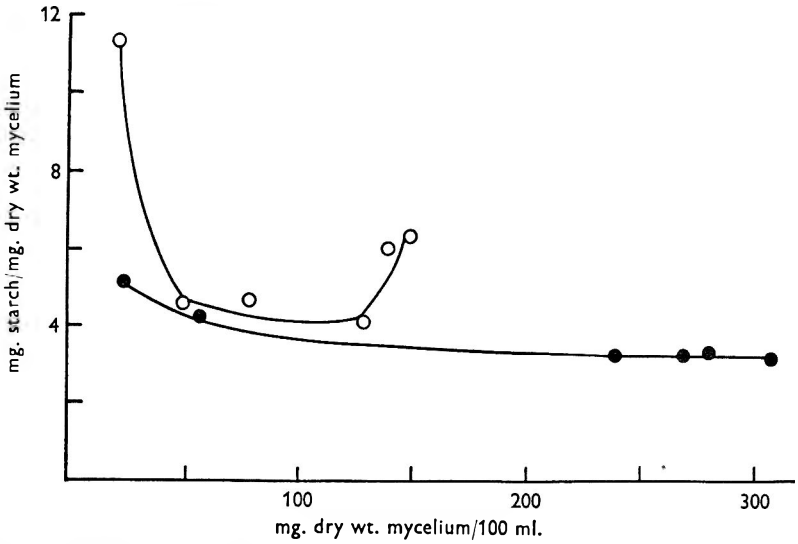


Fig. 7. Specific utilization of starch by *Aspergillus oryzae* at various stages of growth under submerged cultivation using large (●—●) and small (○—○) inocula.

DISCUSSION

In this paper and elsewhere (Meyrath, 1962*a*, 1963; McIntosh & Meyrath, 1963) it has been shown that the effects of inoculum size on various growth properties of *Aspergillus oryzae* can be very pronounced. Use of organisms which react less strongly to a variation in size of inoculum, or use of substrates rich in trace elements (which depress the phenomena described), may have resulted in effects so small that they have been overlooked or left unrecorded by other workers. However, the five other strains of Aspergillus tested did show a response to change in size of inoculum although not quite as strongly as did *Aspergillus oryzae*.

Part of the characteristic response in a small-inoculum culture is likely to be due to a decrease in specific rate of oxygen uptake accompanied by an increase in fermentative reactions (production of ethanol and acids). However, the surplus of ethanol and acids formed is small in comparison with the decreased yield of mycelium in small-inoculum cultures. There are undoubtedly other metabolic reactions which promote the effects caused by size of inoculum. It has to be considered not only how much energy (per unit weight carbohydrate used) is made available, but also whether the released energy is used efficiently. There are strong indications that a young culture from a large inoculum is able to utilize energy for synthetic reactions much more efficiently than an older culture of similar origin or a culture from a small inoculum at any stage of growth. Preliminary tests showed that washed mycelium from large-inoculum cultures in the early exponential phase, when suspended in glucose phosphate buffer, showed a considerable increase in cell material in short-

term tests (1–6 hr.). On the other hand, there was either a decrease or no change in mycelial dry weight when mycelium, similarly treated, was taken from later stages of the exponential phase of large-inoculum cultures, or from any measurable stage of growth of small-inoculum cultures. The higher synthetic ability in young large-inoculum cultures might be due to a greater activity of synthesizing enzymes or perhaps to a more efficient energy transfer from the catabolic to the anabolic enzyme system. This higher assimilation activity, which takes place in a nitrogen-free medium, may lead purely to the formation and accumulation of carbohydrate within the mycelium, as observed originally by Barker (1936) with *Prototheca zopfii*. On the other hand a more orderly increase of cell material might take place by utilization of nitrogenous components present in the cell. Neither possibility has yet been tested for experimentally. In this context it should be noted that the nitrogen content of the mycelium has been shown to be higher at earlier stages of growth (McIntosh & Meyrath, 1963); this nitrogen might serve as reserve material.

Part of this work was carried out at the Department of Agricultural Bacteriology and Fermentation, Swiss Federal Institute of Technology, Zürich. The authors are indebted to Miss A. Mohler and Mr G. Geser (Zürich) and Mr J. McElroy (Glasgow) for careful technical assistance rendered. The stimulating interest of Professor E. O. Morris (Glasgow) is greatly appreciated.

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Size of Inoculum and Nitrogen Metabolism in *Aspergillus oryzae*

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SUMMARY

The influence of size of inoculum on the nitrogen metabolism of *Aspergillus oryzae* in submerged culture was studied. In producing a given weight of mycelium a large-inoculum culture, as compared with a small-inoculum culture, showed a higher degree of assimilation of nitrogen and a lower excretion of nitrogen over most of the growth curve. This comparatively greater efficiency of the large-inoculum culture became more pronounced when mechanical stress in the culture was increased. Under high mechanical stress, the inefficiency of a small-inoculum culture was so marked that in forming unit weight of mycelium it utilized more inorganic nitrogen than did a large-inoculum culture.

INTRODUCTION

Previous work on *Aspergillus oryzae* (Meyrath, 1962, 1963) showed that inoculum size had an important bearing upon multiplication rate in the exponential phase, growth rate in the linear phase and maximum yield. Later (Meyrath & McIntosh 1963), it was shown that the carbon metabolism was less efficient in small-inoculum cultures. In an attempt to discover whether this inefficiency extended to other metabolic processes, the nitrogen metabolism of large-inoculum and small-inoculum cultures was examined under a variety of conditions. This work is reported here.

METHODS

Materials. The strain of *Aspergillus oryzae* used previously (Meyrath, 1962, 1963; Meyrath & McIntosh, 1963) was used again. In all experiments, the inoculum consisted of conidia; the preparation of a conidial inoculum has been described (Meyrath & McIntosh, 1963). The substrate contained per l.: starch, 10 g.; $(NH_4)_2SO_4$, 1 g.; $MgSO_4.7H_2O$, 0·3 g.; Na_2HPO_4, 4·73 g.; KH_2PO_4, 4·54 g.; trace elements: $FeCl_3.6H_2O$, 15 mg.; $CuSO_4.5H_2O$, 50 μg.; $MnSO_4.4H_2O$, 50 μg.; $ZnSO_4.7H_2O$, 500 μg.; $CaCl_2.6H_2O$, 1 mg. (see substrate A 4, Meyrath & McIntosh, 1963).

Procedure. 5 l. quantities of substrate were prepared and maintained at 25° in 6 l. triple-neck flasks fitted with an aerating mechanism, a sampling device and a Vibro-Mix unit (made by AG. für Chemie-Apparatebau, Männedorf, Zürich, Switzerland). The amplitude of the vibrator was adjusted to 2 mm. (high mechanical stress) or 1 mm. (low mechanical stress). The substrate received an inoculum of 2×10^7 conidia/100 ml. (large-inoculum experiments) or 4×10^3 conidia/100 ml. (small-inoculum experiments), the inoculum having a volume of 50 ml. in each case.

The volume of sample taken depended on the mycelial content; samples were usually taken in duplicate or triplicate. The technique for collecting mycelium was described by Meyrath & McIntosh (1963). The mg. dry wt. mycelium in samples from submerged cultures was estimated at intervals to construct a growth curve. At various stages of growth, attention was focussed on three aspects of nitrogen metabolism: organic-N in mycelium, organic-N in culture filtrate, ammonia-N utilized.

Estimation of nitrogen. Where ammonia-N only was to be estimated, concentrated NaOH solution was added to make the mixture alkaline towards phenolphthalein; ammonia was steam distilled and collected in 50 ml. boric acid solution (H_3BO_3

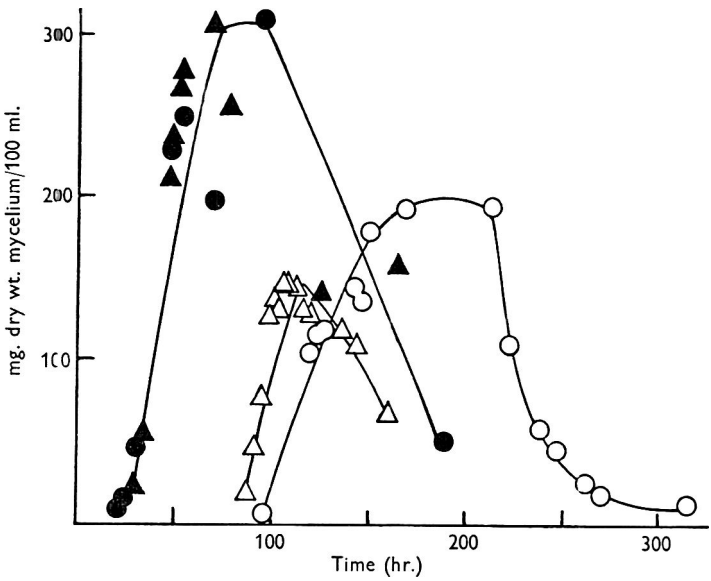

Fig. 1. Pattern of growth of *Aspergillus oryzae*. Large-inoculum cultures were grown under low (●—●) and high (▲—▲) mechanical stress. Small-inoculum cultures were likewise grown under low (○—○) and high (△—△) mechanical stress.

40 g./l.) which was then titrated with 0·02 N-H_2SO_4 using methyl red indicator. Ammonia-N utilized was estimated on 5–20 ml. samples and calculated by subtracting the valne for residual ammonia-N in the culture filtrate from that for initial ammonia-N in the substrate.

Where organic-N or total-N (organic-N and ammonia-N) was to be estimated, digestion was first necessary to convert organic-N to ammonia-N. The material was boiled in micro-Kjeldahl flasks with 2 ml. conc. H_2SO_4 containing traces of copper sulphate and selenium as catalysts until a clear solution was obtained. All the N was then in the form of ammonia-N and was estimated as above. The nitrogen of the mycelium was considered to be in organic form and the pellets were treated accordingly. Organic-N in the culture filtrate was estimated on 5–20 ml. samples and was calculated by subtracting the value for ammonia-N from that for total-N.

RESULTS

The growth curves shown in Fig. 1 compare well with those obtained in earlier experiments. Maximum mycelial yield was greater when a large inoculum was used (Fig. 1). The difference in maximum mycelial yield as between large-inoculum and small-inoculum cultures was more pronounced when the mechanical stress was increased (Fig. 1).

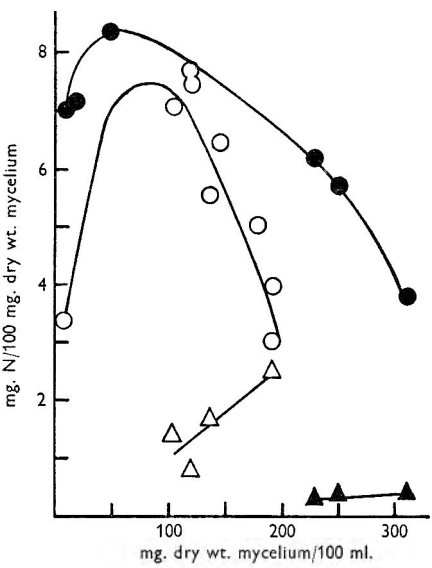

Fig. 2

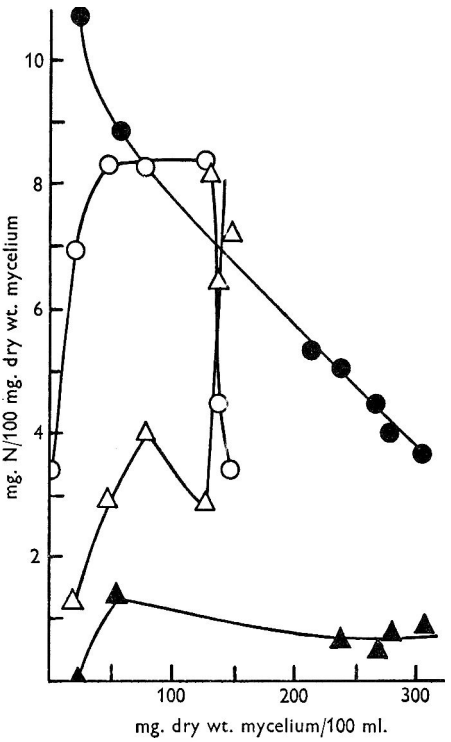

Fig. 3

Figs. 2, 3. Nitrogen incorporated and excreted by *Aspergillus oryzae* at low (Fig. 2) and high (Fig. 3) mechanical stress. Organic-N in mycelium (N incorporated) was estimated in both large-inoculum (●—●) and small-inoculum (○—○) cultures. Organic-N in filtrate (N excreted) was also estimated in both large-inoculum (▲—▲) and small-inoculum (△—△) cultures.

Organic nitrogen in mycelium and in filtrate

Conditions of low mechanical stress are considered first. When organic-N in the mycelium was estimated, a large-inoculum culture showed a high N content in the mycelium in the early stages (Fig. 2); later, there was a steady decrease in this value. In a small-inoculum culture, Fig. 2 shows that the N content was considerably lower in early and late stages and probably had a lower maximum. The amount of organic-N in the filtrate was generally low in a large-inoculum culture (Fig. 2). A small-inoculum culture showed a more pronounced increase in organic-N in the filtrate. From these results at low mechanical stress a pattern of N metabolism may be deduced. The mycelium from a large-inoculum culture is the more efficient, as it

assimilates more N. Further, as organic-N in the filtrate represents N excreted by the mould (for inorganic-N only is present in the initial medium), the results show that a large-inoculum culture excreted very little N. Conversely, in small-inoculum cultures, the lower N content of the mycelium and the higher excretion of organic-N are indicative of inefficiency in N utilization and assimilation.

Corresponding experiments were performed at high mechanical stress (Fig. 3). Organic-N in the mycelium at various stages of growth was examined. Figure 3

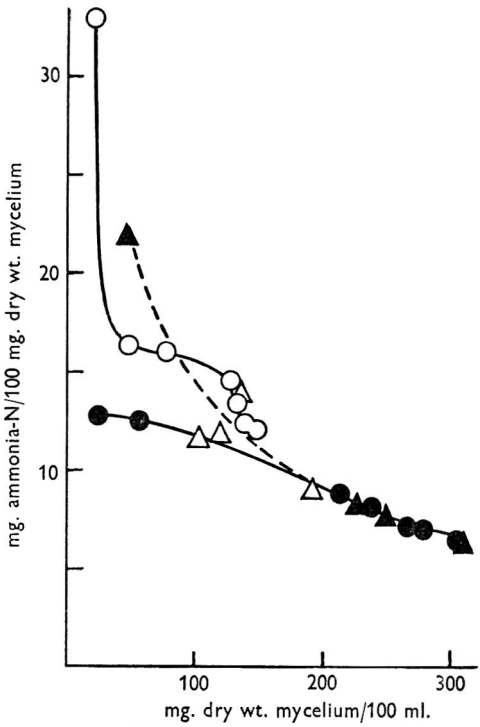

Fig. 4. Ammonia nitrogen metabolized by *Aspergillus oryzae* at low and high mechanical stress. The values for large-inoculum (▲—▲) and small-inoculum (△—△) cultures at low mechanical stress are shown by the broken line. The values for large-inoculum (●—●) and small-inoculum (○—○) cultures at high mechanical stress are shown by the continuous lines.

shows that in a large-inoculum culture the results corresponded very closely to those obtained at low mechanical stress. The small-inoculum mycelium showed a decrease in N content, beginning at about the same point (130 mg. dry wt. mycelium/100 ml.) as at low mechanical stress but this decrease was much more pronounced at high stress. As was found at low mechanical stress, the amount of organic-N in the filtrate was smaller in a large-inoculum culture. A small-inoculum culture showed an increase of organic-N in the filtrate as at low mechanical stress, but the increase was more pronounced (Fig. 3). Thus under conditions of high mechanical stress also, the pattern of assimilation and excretion of N indicates that the large-inoculum culture was the more efficient. It appears, moreover, that at a given point, mycelium of a small-inoculum culture underwent a marked change in metabolic behaviour,

as was shown by a large and rapid excretion of N. This occurred while the mycelium content of the culture was still increasing, just before maximal growth; it did not take place during what is usually called the phase of decline or the autolytic phase. A comparison of Figs. 2 and 3 shows that an increase in mechanical stress accentuated these differences in the behaviour of large-inoculum and small-inoculum cultures.

Metabolism of ammonia-N

At low mechanical stress there was little distinction between a large-inoculum culture and a small-inoculum one in the specific amount of ammonia-N metabolized (see Fig. 4). When, however, the mechanical stress was increased, the small-inoculum culture utilized specifically more ammonia-N than did a large-inoculum culture. Generalizations about the efficiency of a culture cannot safely be made from this particular aspect of N metabolism; efficiency in N metabolism should perhaps be measured not so much by how little N is utilized to produce a given weight of mycelium, as by what is done with the N so utilized. Figure 4 shows that by altering the mechanical stress, the effect of size of inoculum on the amount of ammonia-N utilized was also altered. At low mechanical stress there was no evident influence of inoculum size; at high mechanical stress, the small-inoculum culture metabolized more ammonia-N than did the large-inoculum culture.

DISCUSSION

It should be remembered that in Figs. 2, 3 and 4, points in vertical line represent the same weight of mycelium. The effects, then, are not due to differences in amount of mycelium, but are specific differences. Two cultures of the same mycelium content are not of the same age, because a small-inoculum culture takes longer to reach a given mycelium content than does a large-inoculum culture. That is to say, where points are in vertical line, up to maximal growth, the small-inoculum culture is composed of older mycelium.

The results show that there may be very great changes (increases as well as decreases) in N content of mycelium during culture development. This invalidates the use of N content as sole measure of growth. When there is an increase in dry wt. mycelium, it implies that synthesizing reactions are involved with resultant formation of new cellular substance which can suitably be called 'growth'. It is also doubtful whether the N content of mycelium is a measure of 'living protoplasm' because some of the cellular N might act as storage N; furthermore a comparison of the respiration activity of this mould (Meyrath & McIntosh, 1963) with its cellular N reveals no proportionality. These investigations show that the total synthesizing activity (as expressed by dry wt.) and the specific assimilation of N can be affected by inoculum size.

The decrease in N content during the major part of the development of cultures from large and small inocula was very pronounced and is likely to be due to inefficiency of assimilation. Here again it appears that the cultures underwent an ageing process similar to that described for carbon metabolism (Meyrath & McIntosh 1963); a small-inoculum culture assumed the properties of an old large-inoculum culture when it reached the initial stages of growth of the latter. A decrease in N content of the mycelium during cultivation, although generally less pronounced,

was also observed with other fungi (Cochrane, 1958; Keller, 1952; Schelling, 1952). Taking into consideration the results obtained from several moulds submitted to tests on size of inoculum (Meyrath & McIntosh, 1963), it appears that the described types of effects of size of inoculum among fungi will occur more frequently than might have been anticipated.

We wish to acknowledge the skilled technical assistance given by Mr J. McElroy and to thank Professor E. O. Morris for his interest and encouragement.

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Flagellar Synthesis in *Salmonella typhimurium:* the Incorporation of Isotopically-Labelled Amino Acids into Flagellin

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SUMMARY

A method is described for assaying flagella of *Salmonella typhimurium* based on the incorporation of a ^{14}C-labelled amino acid, mechanical separation of the flagella from the bacteria, and measurement of the radioactivity of the isolated flagella. There is no detectable lag before the appearance of L-[*G*-^{3}H]leucine, or L-[*Me*-^{14}C]methionine in the flagella, and the rates of incorporation of these amino acids into flagella paralleled the rates of incorporation into the general cell protein. Mechanical removal of the flagella did not affect their subsequent rate of formation. The amino acid, ϵ-*N*-methyllysine, present in flagellin, can be labelled with either L-[*U*-^{14}C]lysine or L-[*Me*-^{14}C]methionine. Using ϵ-*N*-methyllysine as a marker, it has not been possible to detect soluble protein precursors in the formation of flagella by *S. typhimurium*.

INTRODUCTION

Although the flagella of *Salmonella typhimurium* are well defined serologically and chemically (Kauffmann, 1954; Ambler & Rees, 1959) and are probably a pure protein named flagellin, studies on their formation have been hampered by the absence of a specific quantitative assay for flagellin. Stocker & Campbell (1959) followed the synthesis of flagella by measuring the total length of flagella in cultures of *S. typhimurium* and found that flagellin behaved as a normal constitutive protein. Mechanical removal of flagella by treating bacteria in a Waring blendor had no effect on the subsequent rate of formation of flagella. Weinstein, Koffler & Moskowitz (1960) reported the presence of flagellin in the soluble protein fraction obtained from lysed spheroplasts of *Proteus vulgaris*. This intracellular flagellin was detected and assayed serologically and amounted to about 1 % of the total flagellin of the culture. The origin of the intracellular material was not determined so it may have arisen during breakage and fractionation of the cells. However, the report of flagellin in the soluble protein fraction of *P. vulgaris* raises the possibility that soluble protein precursors are synthesized during the formation of functional flagella.

The present paper describes possible specific assays of flagella and attempts to demonstrate intermediates in the formation of flagella by *Salmonella typhimurium*. A preliminary report of this work has been given (Kerridge, 1961*a*).

METHODS

Organism and growth media. The organism used throughout this work was *Salmonella typhimurium*, strain sw1061, obtained from Dr T. Iino (National Institute of Genetics, Misimo, Shizuoko-ken, Japan). The culture was maintained on Dorset egg slopes at room temperature. For the preparation of washed suspensions the bacteria were grown at 37° in Roux bottles containing 150 ml. Oxoid nutrient broth, or in 250 ml. conical flasks containing 100 ml. buffered salts solution (DO) + 0·2 % (w/v) glucose (Kerridge, 1959). The bacteria were harvested while still in the exponential phase of growth, washed once with saline (0·85 %, w/v, NaCl) or with distilled water and resuspended at a concentration equivalent to 5 mg. dry wt. organism/ml. Non-flagellated cultures of *S. typhimurium* were obtained by mechanical removal of the flagella (Kerridge, 1959) or by growth of the bacteria at 44° (Kerridge, 1961*b*).

The dry weights of bacteria in suspension were determined from optical density measurements made with a Hilger 'Spekker' absorptiometer and the use of a calibration curve prepared by drying bacterial suspensions of known optical density to constant weight at 105°.

Incorporation of ^{14}C-*labelled amino acids into* Salmonella typhimurium. The incorporation of ^{14}C-labelled amino acids into suspensions of *S. typhimurium* was carried out in DO medium containing 0·2 % (w/v) glucose; the ^{14}C-labelled amino acid was added to give a final concentration of 0·1 μC./ml., although the specific activity varied from experiment to experiment. In certain experiments this medium was supplemented with a mixture of 18 amino acids each at a final concentration of 0·05 mg. of L-isomer/ml. The bacteria were added to a final concentration equiv. 0·5 mg. dry wt/ml. and the suspensions incubated statically at 37°. Occasionally the labelled amino acid was added to cultures of *S. typhimurium* growing exponentially in defined media.

At intervals during incubation, samples containing equiv. 2–3 mg. dry wt. bacteria were pipetted into centrifuge tubes containing sufficient chloramphenicol to give a final concentration of 50 μg./ml. and the tubes cooled to 0°. The bacteria were harvested by centrifugation and, after washing once with cold distilled water, were resuspended in 2·5 ml. distilled water. The flagella were detached mechanically and separated from the bacteria by centrifugation at 5000*g* for 15 min.

Isolation and estimation of ^{14}C-*labelled flagella.* A preparation of unlabelled flagella was added to the supernatant fluid containing the ^{14}C-labelled flagella to a final concentration of 0·1 mg. protein/ml. and the flagella precipitated from this suspension by using the techniques described below:

(*a*) *Precipitation with acriflavin.* This method was derived from the procedure of Bernstein & Lederberg (1955). An aqueous solution of acriflavin was added to the suspension of flagella to a final concentration of 0·05 or 0·1 % (w/v). The tubes were incubated at 37° for 2 hr. and then at 0° for 16 hr. The agglutinated flagella were harvested by centrifugation and the radioactivity determined.

(*b*) *Precipitation with anti-flagellar antiserum.* A volume of 0·04 ml. anti-1.2.3.antiserum (titre 1/10,000; obtained from Dr P. Bradstreet, Serological Standards Laboratory, Central Public Health Laboratory, Colindale Avenue, London, N.W. 9), was added to 2·0 ml. of the flagellar suspension. The tubes were

incubated at 37° for 2 hr. and then at 0° for 16 hr. The antigen + antibody complex was recovered by centrifugation, washed once with saline, and resuspended in 1 ml. 5 % (w/v) trichloroacetic acid (TCA). The precipitate was washed once with 5 % (w/v) TCA and then taken up in N-NH_4OH for the assay of radioactivity.

(*c*) *Precipitation with TCA.* TCA was added to the flagellar suspension to a final concentration of 5 % (w/v) and the suspension stored at 4° for 30 min. After centrifugation and washing with 5 % (w/v) TCA, the precipitate was resuspended in 2 ml. 6 N-HCl and hydrolysed at 105° for 16 hr. The HCl was removed by distillation and samples of the hydrolysate equivalent to 0·1 mg. protein were subjected to electrophoresis on Whatman no. 3MM paper in 0·05 M sodium carbonate + bicarbonate buffer (pH 10·6) for 2 hr. at 20 V./cm. The amino acids were located by dipping the electrophoresis paper in an acidic solution of ninhydrin (ninhydrin, 0·5 g.; acetone, 100 ml.; glacial acetic acid, 30 ml.). The areas corresponding to ε-*N*-methyllysine (NML) and lysine were cut out and the amino acid-ninhydrin complex eluted with water. The eluates were transferred quantitatively to aluminium planchets (8 $cm.^2$) and the radioactivity determined with a thin window gas flow counter (Nuclear Chicago Corp., 333 East Howard Avenue, Des Plaines, Illinois, U.S.A.).

Cell fractionation. The procedure was based on that described by Roberts *et al.* (1955). The deflagellated bacteria were washed once on the centrifuge with distilled water and the pellet resuspended in 2 ml. 5 % (w/v) TCA. After storage at 4° for 2 hr., the precipitated bacteria were harvested by centrifugation and washed once with 5 % (w/v) TCA. The precipitate was extracted for 30 min. at 45° with 2 ml. 75 % (v/v) ethanol in water, the suspension centrifuged, and the pellet extracted for 30 min. at 45° with a mixture of 1 ml. 75 % (v/v) ethanol in water + 1 ml. ether. The ethanol and ethanol + ether extracts were combined and, after the addition of 3 ml. water, were extracted three times with an equal volume of ether to remove lipids. Nucleic acids were extracted from the residue after ethanol + ether extraction with 5 % (w/v) TCA, one extraction at 90° for 30 min. being sufficient to remove the nucleic acid. The TCA-insoluble residue contained the residual protein and cell-wall fractions.

The protein-containing fractions were hydrolysed with 6 N-HCl at 105° for 16 hr. After removal of HCl, the hydrolysate was dissolved in distilled water and samples taken for electrophoresis and for radioactivity assay. Samples were transferred to aluminium planchets (2 $cm.^2$) and the radioactivity determined using a Panax type D657 Scaler (Panax Equipment Ltd., Mitcham, Surrey) in conjunction with a mica end-window Geiger-Muller tube.

Incorporation of L-[*G*-^{3}H]*leucine into* Salmonella typhimurium. L-[*G*-^{3}H]leucine was used in one experiment to follow protein synthesis in *Salmonella typhimurium.* The incubation conditions and fractionation procedure were identical to those used to study the incorporation of ^{14}C-labelled amino acids by *S. typhimurium.* After fractionation the TCA insoluble residues were dissolved in 0·3 ml. of a M solution of Hydroxide of Hyamine 10 – × (Reg. trademark of Rohn and Haas Inc.) in methanol. An aliquot of this solution was added to 1·5 ml. of a liquid scintillator, consisting of 3·5 g. 2,5-diphenyloxazole (PPO) and 50 mg. 1,4-bis-2-(5-phenyloxazolyl) benzene (POPOP) in 1 l. redistilled toluene, contained in a small glass vial. The radioactivity was measured using a Packard Tricarb Liquid Scintillation Spectrometer (Packard Instrument Company, Inc., La Grange, Illinois, U.S.A.).

Disrupted cell preparations. Suspensions of *Salmonella typhimurium* were disrupted by using: (*a*) the Mullard type E 7590B ultrasonic disintegrator and the transducer assembly described by Davies (1959); (*b*) a bacterial press (Hughes, 1951); (*c*) a French pressure cell based on a design supplied by the Department of Terrestial Magnetism, Carnegie Institute, Washington, D.C., U.S.A. The disrupted bacteria were fractionated by centrifugation at 5000*g* for 15 min., followed by 105,000*g* for 120 min., into a fraction containing unbroken bacteria and large particles, a fraction containing mainly ribosomes, and a soluble fraction.

RESULTS

Assay of bacterial flagella. A major difficulty in studying flagellar synthesis is that, up to now, there have been no sensitive methods for quantitative assay of bacterial flagella. Qualitative assays are relatively simple using microscopic examination of living cultures or stained bacterial preparations. In the absence of any specific enzymic activity in the flagellum, a study has been made of the possibility of isolating small quantities of radioactive flagella after their separation from the bacteria. Bernstein & Lederberg (1955) in confirming earlier studies of Sertic & Boulgakov (1936) on the agglutination of salmonella by acridines, reported that it resembled the agglutination of motile bacteria by specific anti-flagellar antiserum and that acridines agglutinated suspensions of isolated flagella. Flagella in phase 2 were agglutinated at a smaller concentration of the acridines than were flagella in phase 1. Addition of acriflavin (final concentration 0·05 % or 0·1 %, w/v) to a suspension of ^{35}S-labelled flagella (flagellar antigen 1.2.3.), kindly provided by Dr R. P. Ambler (Department of Biochemistry, Cambridge) precipitated 80 % of the added flagella (Table 1). Although acriflavin at a fairly high concentration gave good and reproducible precipitation of a purified preparation of flagella, this method was abandoned in favour of precipitation by specific anti-flagellar antiserum.

Table 1. *Precipitation of* ^{35}S-*labelled flagella of* Salmonella typhimurium *with acriflavin*

Suspensions of flagella were incubated with acriflavin for 2 hr. at 37° and then stored for 16 hr. at 4°. The precipitates were harvested by centrifugation, resuspended in N-NH_4OH and the radioactivity determined.

	Flagella (mg./ml.)		
	0·2	0·1	0·05
Acriflavin (%, w/v)	Radioactivity in the precipitate % of the total		
0·1	67	81	82
0·05	77	61	74

Under the experimental conditions used, addition of the specific anti-flagellar antiserum did not completely precipitate the ^{35}S-labelled flagella (Table 2*a*) for not more than 75 % of the added radioactivity was recovered. The results however, were very reproducible (Table 2*b*). Addition of anti-*i* antiserum to the suspension of ^{35}S-labelled flagella lacking this antigen did not cause the formation of any specific complex. In all subsequent serological isolations of isotopically-labelled

flagella, carrier flagella were added to give a final concentration of 0·1 mg. protein/ml. and specific anti-flagellar antiserum (titre 1/10,000) added at a dilution of 1 part in 50.

The incorporation of amino acids into flagella by Salmonella typhimurium. Stocker & Campbell (1959), in a study of the regeneration of flagella by mechanically deflagellated bacteria, found that the increase in total flagellar length, measured in stained preparations, agreed very well with theoretical values calculated on the assumption that flagellin behaved as a constitutive protein. These findings have been confirmed by studying the incorporation of L-[*U*-^{14}C]leucine and L-[*U*-^{14}C]-glutamic acid by cultures of *S. typhimurium* growing exponentially in defined media

Table 2. *Precipitation of* ^{35}S-*labelled flagella of* Salmonella typhimurium *with specific anti-flagellar antiserum*

Specific anti-flagellar antiserum (titre 1/10,000) was added to the flagellar suspensions, the preparations incubated at 37° for 2 hr. and then stored at 4° for 16 hr. The precipitate was removed by centrifugation, washed with saline and TCA (5%, w/v), finally suspended in N-NH_4OH and the radioactivity determined. 92% of the radioactivity in the flagellar preparation was precipitated with 5% (w/v) TCA.

(*a*)

Serum dilution	Flagella (mg./ml.) 0·167	0·125	0·083	0·042
	Radioactivity in the precipitate, % of the total			
1/ 60	64	67	72	67
1/120	38	43	52	62
1/180	32	36	42	60
1/240	24	28	33	48

(*b*)

Serum dilution	Flagella (mg./ml.) 0·2	0·1	0·05	0·025
	Radioactivity in the precipitate, % of the total			
1/50	57	72	74	.
	48	73	75	.
	57	75	74	.
	.	73	73	73

at 37°. Samples were taken at intervals after adding the amino acid, the bacteria washed once with saline and resuspended in 2·5 ml. saline. The flagella were then removed mechanically and separated from the bacteria by centrifugation. The detached flagella were precipitated with specific antiserum and the radioactivity in the precipitated flagella and the residual cell protein determined. The results are shown in Figs. 1 *a* and *b*. There was no difference in the initial rates of incorporation of ^{14}C-labelled amino acid into flagella and into the residual cell protein. Mechanical deflagellation immediately before the addition of the ^{14}C-labelled amino acid had no effect on the relative rates of subsequent incorporation into flagella and residual cell protein.

Growth of *Salmonella typhimurium* at 44° in a complex medium leads to production of non-flagellated bacteria (Quadling & Stocker, 1956, 1962; Kerridge, 1961*a*). If the incubation temperature is lowered from 44° to 37° there is no change in the growth rate of the bacteria and the synthesis of flagella begins after a lag of about one generation time (Kerridge, 1961*a*). Addition of L-[*U*-^{14}C]leucine to the culture immediately after transfer from 44° to 37° led to immediate appearance of ^{14}C in the protein fraction of the bacteria and, after a lag, in the flagella. The rate of incorporation of the leucine in flagella was similar to the rate of incorporation in cell protein (Fig. 2).

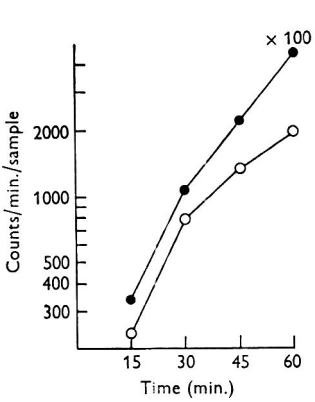

Fig. 1*a*

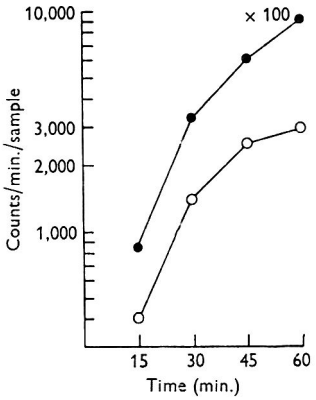

Fig. 1*b*

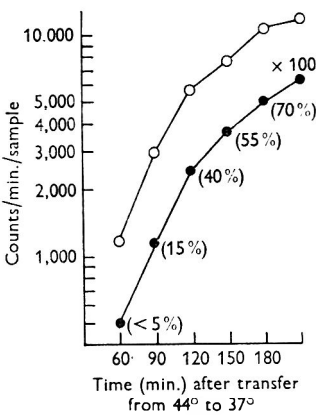

Fig. 2

Figs. 1*a*, *b*. Incorporation of L-[*U*-^{14}C]leucine and L-[*U*-^{14}C]glutamic acid into *Salmonella typhimurium*. L-[*U*-^{14}C]leucine or L-[*U*-^{14}C]glutamic acid (final conc. 0·1 μC./ml.) was added to a culture of *S. typhimurium* growing exponentially in the minimal medium supplemented with a mixture of 18 amino acids (50 μg. of each/ml.). Samples were taken at intervals. The flagella were detached mechanically from the bacteria and precipitated serologically. The incorporation of the amino acid into flagella (-●—●-) and the acid-insoluble residue (-○—○-) was measured.

Fig. 2. Incorporation of L-[*U*-^{14}C]leucine into *Salmonella typhimurium* during recovery of motility at 37° after growth at 44°. *S. typhimurium* growing exponentially at 44° in the minimal medium supplemented with 0·2 % (w/v) Difco vitamin-free casein hydrolysate was harvested by centrifugation and resuspended in the minimal medium supplemented with a mixture of 18 amino acids (50 μg. of each/ml.) and L-[*U*-^{14}C]leucine (0·1 μC./ml.) and incubated aerobically at 37°. After sampling, the flagella were detached mechanically from the bacteria and precipitated serologically. The incorporation of L-[*U*-^{14}C]-leucine into flagella (-●—●-) and the acid-insoluble residue (-○—○-) was measured. The figures in parentheses show % flagellate bacteria determined by microscopic examination of stained bacterial preparations.

Although in the early studies, the formation of flagella by *Salmonella typhimurium* has been considered as an example of the synthesis of a single protein, it is important to remember that the flagella of this organism are organized structures (Kerridge, Horne & Glauert, 1962). The relative rates of incorporation of isotopically-labelled amino acids into flagella and the general cell protein show that flagellin is a constitutive protein, but give no indication of possible protein intermediates between free amino acids and the functional flagellum. If such intermediates exist, they should be detectable by suitable isotopic techniques.

No evidence was obtained for the existence of a precursor pool from experiments

involving the addition of 200 times excess of unlabelled leucine to a culture of *Salmonella typhimurium* growing exponentially in a defined medium containing L-[*U*-^{14}C]leucine. The incorporation of L-[*U*-^{14}C]leucine into the residual protein fraction and the flagella ceased after adding the unlabelled amino acid (Fig. 3). However, this technique would only reveal a large, not a small pool of protein intermediates. The possibility that a small pool exists was next studied using high specific activity L-[*G*-^{3}H]leucine. Samples were taken within 2 min. of adding leucine, but extrapolating to zero time gave no indication of a lag in the incorporation of tritium into flagella (Fig. 4) and therefore it is unlikely that a precursor pool capable of supporting more than a few sec. synthesis of flagella is present in *S. typhimurium*.

Table 3. *Distribution of flagellin in the soluble protein fraction of* Salmonella typhimurium

Bacterial suspensions were disrupted mechanically and the presence of flagellin in the 100,000*g* supernatant fraction detected serologically.

Growth temp. (°)	Motility	Method of cell breakage	Flagellin in the 100,000*g* supernatant fluid
37	+	French pressure cell	+
37	− (Flagella detached mechanically)	French pressure cell	+
44	−	French pressure cell	−
37	+	Hughes press	+
37	+	Lysis of penicillin-induced spheroplasts	+
Control flagellar suspension		French pressure cell	+

Serological examination of the soluble protein fraction of Salmonella typhimurium *for the presence of flagellin.* Weinstein *et al.* (1960) reported that flagellin could be detected in the soluble protein fraction of *Proteus vulgaris*. Attempts were made to repeat this finding with *Salmonella typhimurium* and to extend it by including methods of cell breakage other than lysis of penicillin-induced spheroplasts. Suspensions of *S. typhimurium* were disrupted mechanically by treatment in a bacterial press (Hughes, 1951), in a French pressure cell, and by the lysis of penicillin-induced spheroplasts. The broken cell preparations were centrifuged at 105,000*g* for 120 min. and the supernatant fraction freeze-dried. The soluble proteins were resuspended in saline (0·85 %, w/v) and examined serologically by the agar diffusion technique (Ouchterlony, 1953); the results are shown in Table 3. In all cases where flagellated bacteria were disrupted mechanically, flagellin was detected in the soluble protein fraction. A control experiment in which a suspension of isolated flagella, at a concentration equivalent to that present in the bacterial suspension, was 'disrupted' in a French pressure cell and examined serologically after centrifugation at 105,000*g* for 120 min. cast some doubt on the validity of the results, since 'flagellin' could obviously have arisen during mechanical disintegration of the bacteria. Mechanical removal of flagella before breaking the bacteria did not eliminate 'flagellin' from the soluble protein fraction, but, as it is difficult to remove all the flagella by this procedure it is impossible to decide conclusively if the flagellin present in the soluble protein fraction is an artifact. If the flagellin

detected in the soluble fraction is assumed to be a precursor for flagella, then the amount present (about 1 % of the total flagellin) is sufficient for synthesis for about 1 min. during normal exponential growth.

Incorporation of L-[*Me*-^{14}C]*methionine into* Salmonella typhimurium. In their study of the chemical structure of flagellin from *S. typhimurium*, strain sw 1061, Ambler & Rees (1959) found the unusual amino acid, ε-*N*-methyllysine (NML), an amino acid not previously reported in any bacterial protein. This amino acid can be labelled by incubating cultures of *S. typhimurium* with either L-[*U*-^{14}C]lysine or L-[*Me*-^{14}C]methionine. The general pattern of labelling of *S. typhimurium* following incubation of the bacteria in a minimal medium containing L-[*Me*-^{14}C]methionine is shown in Table 4. More than 95 % of the activity in the flagella was recovered as NML. The incorporation of the methyl group from L-[*Me*-^{14}C]methionine into cellular fractions other than flagella was considered as a possible method for studying the initial stages and possible intermediates in the formation of flagella by *S. typhimurium*.

Table 4. *Distribution of* ^{14}C *in cellular fractions of* Salmonella typhimurium *after incorporation of* L-[*Me*-^{14}C]*methionine*

A suspension of *S. typhimurium* was incubated in the minimal medium supplemented with L-[*Me*-^{14}C]methionine (0·05 μC./ml., 10 μg./ml.) After incubation for 4 hr. at 37° the bacteria were harvested by centrifugation and washed with distilled water. The suspension was divided into two parts, of which one was fractionated by a method based on that of Roberts *et al.* (1955), while the other was disrupted by ultrasonic vibration and fractionated by centrifugation. The radioactivity of the various fractions was determined.

Fraction	Radioactivity as % of the total
	Chemical fractionation
'Pool'	1·6
Ethanol soluble protein	4·3
Lipid	14
Nucleic acid	5
Acid insoluble residue	75·1
	Physical fractionation
10,000*g* pellet	22·2
100,000*g* pellet	28
Soluble fraction (includes 'pool')	49·8

Addition of L-[*Me*-^{14}C]methionine to a suspension of *Salmonella typhimurium* incubated in minimal medium resulted in labelling the flagellar NML and of other cell constituents (Fig. 5). No samples were taken earlier than 2 min. after adding methionine, but extrapolation of the curve to zero time gave no indication of an appreciable lag before the rate of incorporation of the methyl group from methionine in the flagellar NML reached its maximum value. Incorporation in NML paralleled incorporation in the TCA-insoluble residue and gave a satisfactory measure of the rate of synthesis of flagellin. Mechanical removal of the flagella immediately before adding methionine did not affect the relative rates of labelling of flagella and residual protein.

Salmonella typhimurium has the same growth rate in a complex medium at 44° as at 37°, but at 44° the synthesis of flagella is inhibited and on continued incuba-

tion a non-flagellate culture is obtained. The rates of incorporation of L-[*Me*-^{14}C]-methionine in the residual protein fractions by bacteria previously grown at 37° were the same at 44° as at 37° (Fig. 6*a*), but the rate of labelling of flagellar NML was markedly decreased after incubation for 5 min. at 44° as compared with the rate at 37° (Fig. 6*b*). The results obtained for the methyl labelling of flagellar ε-*N*-methyl-lysine were consistent with those obtained by using incorporation of other isotopically-labelled amino acids to follow flagellar synthesis, and this technique provided a satisfactory method for determining the rate of flagellar formation by *S. typhimurium*.

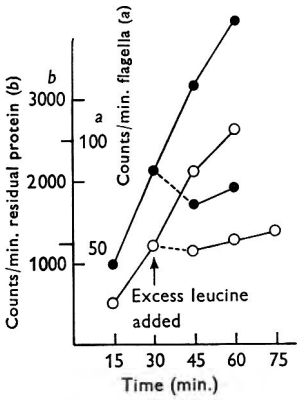

Fig. 3

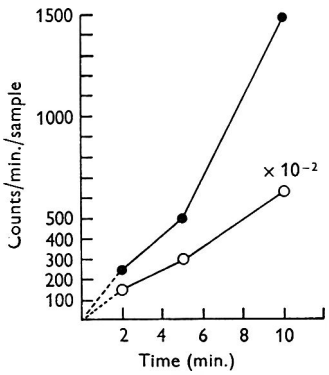

Fig. 4

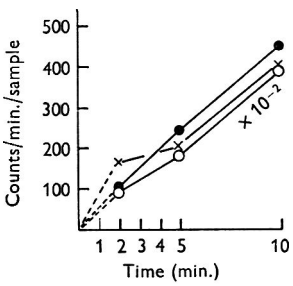

Fig. 5

Fig. 3. The effect of adding excess unlabelled leucine on the uptake of L-[*U*-^{14}C]leucine by *Salmonella typhimurium*. L-[*U*-^{14}C]leueine (final conc. 0·1 μC./ml.) was added to a culture of *S. typhimurium* growing exponentially in the minimal medium supplemented with a mixture of 18 amino acids (50 μg. of each/ml.). After 30 min. incubation at 37° the culture was divided into two and, to one portion, unlabelled leucine (final cone. 5 mg./ml.) was added. Samples were taken at intervals, the flagella were detached from the bacteria and precipitated serologically. The radioactivity of flagella (-●—●-) and the acid-insoluble residue from the deflagellated bacteria (-○—○-) was measured.

Fig. 4. Incorporation of L-[*G*-^{3}H]leucine into exponentially growing *Salmonella typhimurium*. L-[*G*-^{3}H]leucine (final conc. 1·6 μC./ml.; specific activity 105 μC./μM) was added to a culture of *S. typhimurium* growing exponentially in the minimal medium supplemented with a mixture of 18 amino acids (50 μg. of each/ml.). Samples were taken at intervals and the flagella were detached mechanically from the bacteria and precipitated serologically. The incorporation of L-[*G*-^{3}H]leucine into the flagella (-●—●-) and the acid insoluble residue of the bacteria (-○—○-) was determined.

Fig. 5. Incorporation of L-[*Me*-^{14}C]methionine into *Salmonella typhimurium*. *S. typhimurium* growing exponentially in the minimal medium was harvested by centrifugation and resuspended in the same medium supplemented with L-[*Me*-^{14}C]methionine (2·3 μg./ml.; 0·1 μC./ml.) and incubated at 37°. After sampling, the flagella were detached mechanically from the bacteria. The flagella and the acid-insoluble residue were hydrolysed with 6N-HCl at 105° for 16 hr. and after removal of the HCl, aliquots were electrophoresed at pH 10·6. The material corresponding to NML was eluted and its radioactivity determined. -●—●-, NML in the flagella; -×—×-, 'NML' in the acid-insoluble residue; -○—○-, total activity in the acid insoluble residue.

Flagellar precursors in Salmonella typhimurium. Suspensions of *Salmonella typhimurium* were incubated in the minimal medium supplemented with L-[*Me*-^{14}C]-methionine, and, after removal of the flagella by treatment in the Waring blendor followed by centrifugation, were precipitated with TCA (final concentration 5 %,

w/v). The residual protein fraction was hydrolysed with 6 N-HCl and the resulting hydrolysate electrophoresed in the presence of carrier NML. The areas corresponding to NML were cut out and the amino acid-ninhydrin complexes eluted and their radioactivity determined. The results of a typical experiment are shown in Fig. 5. The 'NML' of this fraction was labelled at approximately the same rate and to the same extent as the flagellar NML. The quantity of 'NML' detected in the deflagellated bacteria was much greater than expected from the values of 'intracellular flagellin' determined serologically. Similar results were obtained using L-[*U*-^{14}C]lysine as the precursor of 'NML' (Table 5).

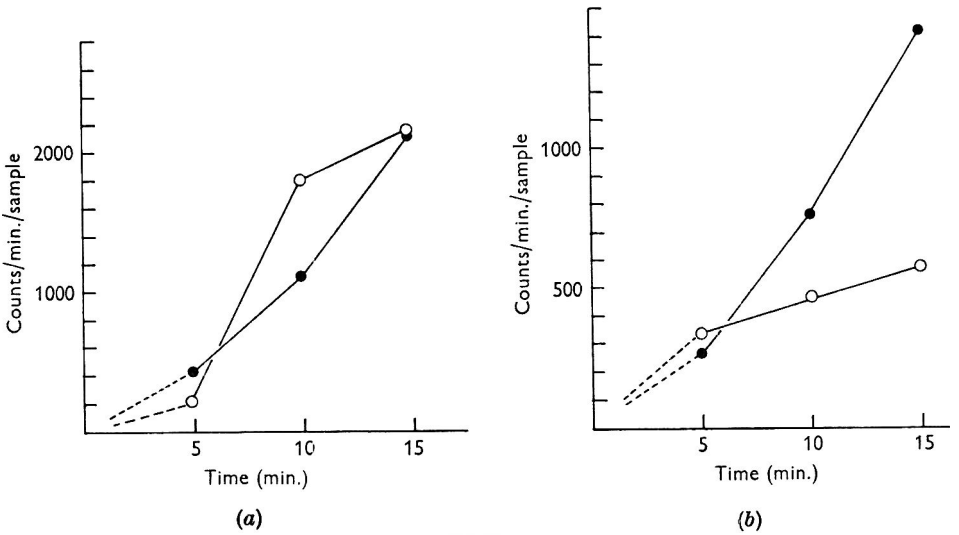

Fig. 6. Incorporation of L-[*Me*-^{14}C]methionine into *Salmonella typhimurium*: (*a*) incorporation into the acid-insoluble material; (*b*) incorporation into flagellar NML. *S. typhimurium* growing exponentially at 37° was harvested and resuspended in the minimal medium supplemented with L-[*Me*-^{14}C]methionine (2·3 μg./ml.; 0·1 μC./ml.) and incubated at either 37° or 44°. After sampling, the flagella were detached mechanically and separated by centrifugation from the bacteria. The acid-precipitated flagella were hydrolysed and the hydrolysate electrophoresed at pH 10·6. The material corresponding to NML was eluted and the radioactivity assayed. The incorporation of L-[*Me*-^{14}C]methionine into the acid insoluble residue was also measured. -●—●-, Bacteria incubated at 37°; -○—○-, bacteria incubated at 44°.

The amount of L-[*Me*-^{14}C]methionine present in the incubation medium was sufficient for incorporation to occur at the maximum rate for about 15–20 min. and subsequently the rate rapidly decreased. When incubation is continued for longer periods, any precursors labelled during the first 15 min. will be incorporated in flagella which will increase in radioactivity at the expense of the precursor fraction. Alternatively, an excess of unlabelled methionine can be added after the first 15 min. incubation. Addition of carrier-free L-[*U*-^{14}C]lysine or L-[*Me*-^{14}C]methionine to suspensions of *Salmonella typhimurium* incubated in minimal medium resulted in labelling of the flagellar NML and also 'NML' in both the ethanol-soluble protein and the residual protein fractions (Fig. 7*a*, *b*). In neither case was there any evidence for a decrease in the radioactivity of 'NML' in either the ethanol-soluble

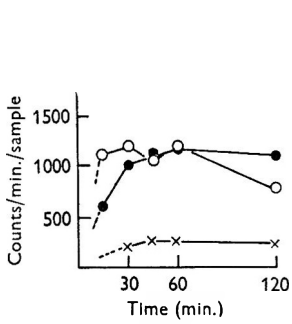

Fig. 7*a*

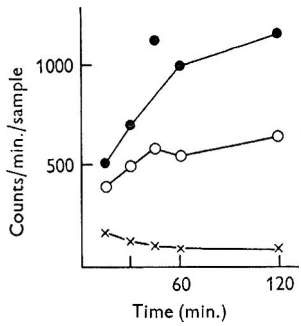

Fig. 7*b*

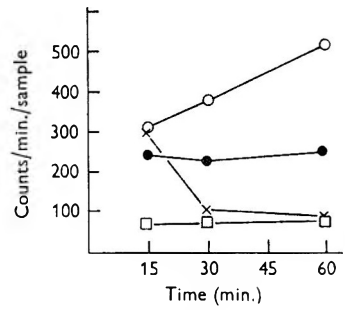

Fig. 8

Fig. 7. Incorporation of L-[*U*-^{14}C]lysine (*a*) and L-[*Me*-^{14}C]methionine (*b*) into 'NML' by *Salmonella typhimurium*. *S. typhimurium* growing exponentially in the minimal medium was harvested and resuspended in the same medium supplemented with either L-[*U*-^{14}C]lysine (1·7 μg./ml.; 0·1 μC./ml.) or L-[*Me*-^{14}C]methionine (2·3 μg./ml.; 0·1 μC./ml.) and incubated at 37°. Samples were taken at intervals and the flagella removed mechanically. The deflagellated bacteria were fractionated into an ethanol-soluble and a residual protein fraction. These fractions were hydrolysed and the hydrolysates electrophoresed at pH 10·6 with an NML marker. The NML-ninhydrin complexes were eluted and the radioactivity determined. -●—●-, Flagella; -×—×-, ethanol soluble protein; -○—○-, residual protein.

Fig. 8. The effect of addition of unlabelled methionine on the distribution of ^{14}C in cellular fractions of *Salmonella typhimurium* previously labelled with L-[*Me*-^{14}C]-methionine. *S. typhimurium* growing exponentially in the minimal medium was harvested and resuspended in the same medium supplemented with L-[*Me*-^{14}C]methionine and incubated at 37°. After 15 min. incubation at 37°, unlabelled methionine was added (final conc. 100 μg./ml.). Samples were taken at intervals, the flagella removed mechanically and the deflagellated bacteria disrupted ultrasonically. The disintegrated bacteria were fractionated by centrifugation into a low speed pellet (P_1), a high speed pellet (P_2), and a supernatant fraction (S). The proteins from these fractions were hydrolysed and the resulting amino acids separated electrophoretically. The NML-ninhydrin complexes were eluted and their radioactivity determined. -●—●-, Flagellar protein; -□—□-, low-speed pellet; -×—×-, high-speed pellet; -○—○-, supernatant fraction.

Table 5. *The distribution of* ^{14}*C in cellular fractions of* Salmonella typhimurium *after the incorporation of* L-[*U*-^{14}*C*]*lysine or* L-[*Me*-^{14}*C*]*methionine*

Suspensions of *S. typhimurium* were incubated in the minimal medium supplemented with either L-[*U*-^{14}C]lysine (0·1 μC./ml.; 1·7 μg./ml.) or L-[*Me*-^{14}C]methionine (0·1 μC./ml.; 2·3 μg./ml.). After 15 min. incubation, the bacteria were harvested by centrifugation, the flagella removed mechanically and the deflagellated bacteria disrupted ultrasonically. The disintegrated bacteria were fractionated by centrifugation at 10,000*g* for 15 min. and 100,000*g* for 120 min. into a low speed pellet (P_1), a high-speed pellet (P_2) and a supernatant fraction (S). The acid-insoluble protein in these fractions was hydrolysed with 6 N-HCl and, after electrophoresis of the hydrolysate, the radioactivity of the 'NML' and the lysine was determined.

	Cellular fraction							
	Flagella		P_1		P_2		S	
Amino acid	NML	Lys.	NML	Lys.	NML	Lys.	NML	Lys.
Precursor amino acid	Radioactivity, counts/min./mg. dry wt bacteria							
L-[*U*-^{14}C]lysine	370	570	36	670	88	6450	160	11640
L-[*Me*-^{14}C]methionine	396	.	28	.	97	.	150	.

protein or the residual protein fractions corresponding to an increase in the flagellar NML. It is unlikely therefore that either fraction contains an appreciable amount of protein acting as a precursor for flagella.

The above chemical fractionation is arbitrary and a more satisfactory procedure was then adopted, based on the centrifugal separation of cell constituents after ultrasonic disintegration. The distribution of L-[*Me*-^{14}C]methionine and L-[*U*-^{14}C]-lysine in the 'NML' and lysine of the various cell fractions of *Salmonella typhimurium* after incubation in the minimal medium supplemented with the amino acid is shown in Table 5. Essentially the same pattern of labelling occurred in both cases and 'NML' was detected in all fractions. Addition of excess of unlabelled amino acid after 15 min. incubation in the presence of L-[*Me*-^{14}C]methionine caused a decrease in the labelling of the 'NML' in the ribosome fraction equivalent to the increase in the labelling of 'NML' of the soluble protein fraction, but there was no corresponding increase in the radioactivity of flagellar NML (Fig. 8).

Preliminary electrophoretic separation of the proteins present in the soluble fraction indicated that the 'NML' was not confined to one particular protein but was distributed throughout many, if not all, the proteins present in this fraction.

DISCUSSION

The methods for flagellar assay described in this paper involve the incorporation of a radioactive amino acid followed by measurement of the radioactivity of the isolated flagella after separating them from the bacteria. It is impossible to remove flagella completely from bacteria but, by standardizing the experimental conditions, the number of flagellar 'stumps' remaining on the bacteria should be small and reasonably constant. Complete separation of the flagella from the deflagellated bacteria is difficult to achieve except by centrifugation at high speeds and a number of viable bacteria will be present in the supernatant fluid after centrifugation. An allowance must therefore be made for possible errors arising from these bacteria. Precipitation of flagella by adding TCA gives a measure of the radioactivity in both flagella and contaminating bacteria, and up to 5% of the radioactivity in the precipitate may be due to the presence of bacteria. The presence of short stumps of flagella on bacteria can result in their precipitation by anti-flagellar antiserum and also bacteria may be trapped in the antigen-antibody complex, so increasing the potential error of the assay.

The finding by Ambler & Rees (1959) that NML is present in the flagellin of *Salmonella typhimurium* strain SW1061, but not in appreciable amounts in its general cell protein suggested a more specific method of flagellar assay. NML was isotopically labelled with either L-[*U*-^{14}C]lysine or L-[*Me*-^{14}C]methionine and, after hydrolysis of the detached flagella, NML could be separated electrophoretically and its radioactivity determined. The percentage of radioactivity in NML due to other substances present in the precipitate is lower than the possible contamination in the other methods of flagellar assay. This method is now used routinely for studying the formation of flagella by *S. typhimurium*. The subsequent finding that the incorporation of either L-[*U*-^{14}C]lysine or L-[*Me*-^{14}C]methionine by *S. typhimurium* led to labelling of a compound present in the protein fraction of deflagellated bacteria which behaved electrophoretically as NML, did not invalidate the

method, since even the maximum contamination due to bacteria in the flagellar suspension was negligible.

The relative rates of amino acid incorporation into the flagella and the general cell protein agree very well with the findings of Stocker & Campbell (1959) and Quadling & Stocker (1962), with one exception. The incorporation of methionine in flagella of *Salmonella typhimurium* incubated at 44° decreased rapidly although incorporation into the general cell protein continued at the same rate as in the control culture, whereas Quadling & Stocker (1962) found that synthesis of flagella continued for about one generation time at 44°. This discrepancy is probably due to the fact that these authors used a complex medium, whereas the incorporation of L-[*Me*-^{14}C]-methionine was studied in a glucose + ammonium salts medium in which *S. typhimurium* cannot grow at 44°.

The fact that flagellin is detectable in the soluble protein fraction of both *Proteus vulgaris* (Weinstein *et al.* 1960) and *Salmonella typhimurium* raised the question of possible soluble protein intermediates in the formation of functional flagella by these organisms. There are, of course, other possible explanations for the presence of flagellin within the cell: for example, the flagellin could merely be a by-product of the synthetic pathway, or might arise from breakdown of flagella during disruption of the bacteria. However, the finding that flagella may be degraded during cell breakage does not exclude the possibility that a fraction of the 'intracellular flagellin' is acting as an intermediate in the formation of the flagella.

The absence of a lag before the appearance of either L-[*G*-^{13}H]leucine or L-[*Me*-^{14}C]-methionine in the flagella of *Salmonella typhimurium* after adding the amino acid to the culture suggests that any pool of intermediates is small. A lag of about 1 min. would be expected before the rate of incorporation reached its maximum value if all the flagellin in the soluble protein fraction was to act as an intermediate. No lag of this type was ever detected in the incorporation experiments.

The labelling of the flagellar NML by L-[*Me*-^{14}C]methionine provided a satisfactory measure of the rate of flagellar formation by *Salmonella typhimurium* and it was hoped that tracing NML in other cellular fractions might provide evidence either for or against flagellar precursors. The quantity of 'NML' detected in deflagellated bacteria after incorporation of either L-[*U*-^{14}C]lysine or L-[*Me*-^{14}C]-methionine was considerably greater than the value expected from the serological assay of 'intracellular flagellin'. This intracellular 'NML' was not isolated and chemically characterized, but the isotopic labelling and its electrophoretic behaviour were identical with those of flagellar NML. The possibility that the 'NML' containing material present within the bacterium, or some part of it, was acting as a flagellar precursor was studied by using pulse-labelling and chasing with the unlabelled amino acid. In no case was it possible to show that the radioactivity of the flagellar NML increased concomitantly with a decrease in the radioactivity of 'NML' in any fraction of the bacteria. Preliminary electrophoretic separation of proteins from the soluble fraction of disrupted bacteria indicated that 'NML' may be present in trace amounts in a number of bacterial proteins and is not confined to flagellin. Stocker, McDonough & Ambler (1961) suggested that the methylation of lysine may occur after its incorporation in the polypeptide chain of flagellin, in which case the small amount of 'NML' detected in other cellular proteins could be due to a lack of absolute specificity of the methylating system for flagellin. The

failure to find any soluble protein intermediates in the formation of flagella does not preclude the possibility that such precursors occur in extremely small amounts. The complete absence of soluble intermediates would imply that the ribosomes synthesizing flagellin molecules are located at the site of aggregation of the individual molecules to form the functional flagellum.

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Cultural and Serological Studies on *Haemophilus vaginalis*

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SUMMARY

When several strains of *Haemophilus vaginalis* were grown on Casman rabbit blood agar, individual morphological and cultural differences were noted between the Amies strains which formed pleomorphic and filamentous organisms and large, umbonate colonies, and the Dukes, Edmunds, King and U/L strains which were microscopically coccobacillary to bacillary, non-filamentous, and formed minute convex smooth colonies. Dukes, Edmunds, King and U/L strains required whole blood for maintenance while a whole blood derivative, e.g. peptic digest or Difco chocolate-yeastolate agar, was sufficient for the maintenance of the Amies strains. Serological studies by tube agglutination, direct, indirect and inhibition immunofluorescent methods showed that Dukes, Edmunds, King and U/L strains reacted in a homologous manner with *H. vaginalis* antisera nos. 317, 394 and 4984. Amies strains did not react with these antisera. However, Amies strains cross-reacted with *H. aegyptius* antiserum 180A, while the Dukes, Edmunds, King and U/L strains did not react with this antiserum.

INTRODUCTION

Several investigators (Leopold, 1953; Gardner & Dukes, 1954, 1955; Gardner, Dampeer & Dukes, 1957; Amies & Jones, 1957; Brewer, Halpern & Thomas, 1957; Edmunds, 1960*a*; Lapage, 1961; and others) have reported finding a small Gram-negative bacillus in the human female and male genito-urinary tract. These investigators used various media for the isolation and cultivation of this organism, named *Haemophilus vaginalis* by Gardner & Dukes (1955). Although most of these investigators used the criteria for identification established by Gardner & Dukes, several reports were at variance with the cultural behaviour and staining characteristics. Amies & Jones (1957) noted smooth–rough (S–R) colony forms of their strains, and their rough colonies contained more filamentous forms of the organism than did the smooth. No other investigators have reported S–R dissociation of *H. vaginalis*, nor have they reported filamentous forms. Variability in Gram reaction was observed by Brewer *et al.* (1957), Lutz & Burger (1957) and Edmunds (1960*a*). Dukes & Gardner (1961) reported Gram variability in 72 and 90 hr. cultures and associated this with the formation of volutin granules. Edmunds (1960*a*) related Gram variability to volutin granules which he believed to be formed by the fermentation of certain sugars, in particular glucose. Later he found (1960*b*) that haemolytic activity

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varied with the animal source of red blood cells. On his meat digest agar he obtained optimal haemolysis with human red blood cells whilst lysis of rabbit red blood cells was variable. Dukes & Gardner (1961) observed a green to brown discoloration type of haemolysis on Casman blood agar medium to be produced by most of the strains investigated. In view of these differences it was decided to make a comparative cultural study of representative strains and to determine whether serological studies might bear out the cultural differences. Strains of *H. aegyptius* and *H. influenzae* were also included in the study.

METHODS

Organisms used

Haemophilus vaginalis: strains 317, 394, 513 and 594 from Dr C. D. Dukes; strains H36, H76, H2030 from Dr C. R. Amies; strain T145 from Dr P. N. Edmunds; strain 4984 (King strain) from Miss E. O. King; strains 342 and 867 (U/L strains) from University of Louisville.

Haemophilus aegyptius: strains 180A and 181A from Dr M. Pittman.

Haemophilus influenzae: ATCC strains 8412, 9006, 9007, 9008 and 9334 from the American Type Culture Collection.

Media. Casman (1947) broth and agar (Difco) + 5 % fresh defibrinated rabbit blood. Chocolate-yeastolate (Difco) agar (5 % defibrinated rabbit blood and 1 % yeastolate added to heart infusion agar base). Fildes peptic digest agar (1920) with defibrinated rabbit blood and Casman agar or heart infusion agar.

Incubation was at 37° in a candle jar.

Staining. Smears of *Haemophilus vaginalis* cultured for up to 5 days in Casman rabbit blood agar were prepared, air dried and heat-fixed. The smears were stained by the potassium mercuric iodide modification of the Gram method, by Giemsa and by basic fuchsin dilute solution. Strains of a vaginal diphtheroid and of *Staphylococcus aureus* were used as Gram-positive controls.

Antiserum preparation. Antisera for *Haemophilus vaginalis* strains 317, 394 and 4984, and for *H. aegyptius* strain 180A were prepared by inoculating live organisms ($3–4 \times 10^6$ bacteria/ml. sterile saline) intravenously, and formalin-killed organisms mixed with Freund adjuvant (Difco) subcutaneously, into the heel pad of normal, healthy New Zealand male rabbits (4–6 lb.) at various times during 24 days. Antisera were obtained one week following the last inoculation by sterile cardiac puncture, and stored at −60° for subsequent use. Antisera for *H. vaginalis* strains 317 and 4984 and for *H. aegyptius* strain 180A were used to prepare fluorescein-conjugated globulins by the method of Cherry, Goldman, Carski & Moody (1960). Human liver powder and bovine red bone marrow were used for the adsorption of the globulin conjugates to decrease non-specific fluorescence. Immunofluorescent reactions were carried out by direct, indirect and inhibition methods on air-dried heat-fixed bacterial smears. The fluorescence equipment used in these studies had an HB–200 mercury vapour bulb as a light source, with two Corning 5840 exciter filters in one case, and a BG12 exciter filter in the other.

Agglutination tests. Tube agglutination tests (Evans, 1957) were done with live bacteria of various species and strains derived from 18 hr. cultures. The diluent was Hanks balanced salt solution containing 0·1 % bovine serum albumin. The tubes

were incubated in a 37° water bath for 3 hr. and examined macroscopically and microscopically for agglutination. The tubes were then held at 4° for 18 hr., and again examined for agglutination.

RESULTS

Morphology and staining reactions

Smears made from 18 hr. Casman rabbit blood agar cultures showed a bacillus ranging from coccobacillary to bacillary forms for the Dukes, King, Edmunds and U/L strains of *Haemophilus vaginalis*; the three Amies strains when grown on Casman medium showed extreme pleomorphism, with many filamentous forms. The

Table 1. *Growth of various strains of* Haemophilus vaginalis

Strain	Casman agar	Casman rabbit blood agar	Casman rabbit blood broth	Fildes peptic digest agar	Chocolate yeastolate agar
Dukes 513	−	+	+	−	±
Dukes 594	−	+	+	−	±
Dukes 394	−	+	+	−	±
Edmunds T145	−	+	+	−	±
King 4984	−	+	+	−	±
U/L 867	−	+	+	−	±
U/L 842	−	+	+	−	±
Amies H76	−	+	±	+	+
Amies H36	−	+	±	+	+
Amies H2030	−	+	±	+	+
Pittman 180A*	−	+	−	+	+

+, Growth; ±, sparse growth (could not be subcultured); −, no growth.
* a strain of *H. aegyptius*

Table 2. *Tube agglutination reaction*

	Reciprocal of agglutination titre with antisera							
	H. vaginalis 317		*H. vaginalis* 4984		*H. vaginalis* 394		*H. aegyptius* 180A	
Antigen	3 hr.	18 hr.	3 hr.	18 hr.	3 hr.	18 hr.	3 hr.	18 hr.
H. vaginalis 317 (Dukes)	5120	5120	1280	1280	640	640	10	10
H. vaginalis 513 (Dukes)	640	640	320	640	320	320	0	0
H. vaginalis 394 (Dukes)	640	640	320	320	2560	2560	10	10
H. vaginalis 594 (Dukes)	320	320	640	640	640	640	0	0
H. vaginalis 4984 (King)	640	640	5120	5120	320	320	0	0
H. vaginalis T145 (Edmunds)	320	320	640	1280	640	1280	0	0
H. vaginalis 842 (U/L)	640	640	320	320	320	320	0	0
H. vaginalis 867 (U/L)	160	320	1280	1280	320	640	0	0
H. vaginalis H2030 (Amies)	10	10	10	10	10	10	40	80
H. vaginalis H76 (Amies)	20	10	10	0	0	0	40	80
H. aegyptius 180A (Pittman)	0	10	10	10	0	0	2560	2560
H. aegyptius 181A (Pittman)	0	0	0	0	0	0	1280	1280
H. influenzae 9008 (ATCC)	0	0	0	0	0	0	0	0
H. influenzae 8412 (ATCC)	0	0	0	0	0	0	0	0
H. influenzae 9006 (ATCC)	0	0	0	0	0	0	0	0
H. influenzae 9334 (ATCC)	0	0	0	0	0	0	0	0
H. influenzae 9007 (ATCC)	0	0	0	0	0	0	0	0

Table 3. *Inhibition immunofluorescent reactions*

Antigen	Non-fluorescent *H. vaginalis* antiserum 317 dilutions								Non-fluorescent *H. vaginalis* antiserum 4984 dilutions							
	0	10	20	40	80	160	320	640	0	10	20	40	80	160	320	640
H. vaginalis 4984 (King)	+	+	+	±	−	−	−	−	+	+	+	+	+	+	±	−
H. vaginalis 317 (Dukes)	+	+	+	+	+	±	±	−	+	+	+	±	−	−	−	−
H. vaginalis 394 (Dukes)	+	+	+	±	−	−	−	−	+	+	+	±	±	−	−	−
H. vaginalis 842 (U/L)	+	+	+	±	−	−	−	−	+	+	+	+	±	−	−	−
H. vaginalis T 145 (Edmunds)	+	+	+	+	±	−	−	−	+	+	+	+	+	±	±	−
H. aegyptius 180 A (Pittman)	±	−	.	.	.	.	.	.	±	−	.	.	.	.	.	.

Antigen	Non-fluorescent *H. vaginalis* antiserum 394* dilutions								Non-fluorescent *H. aegyptius* antiserum 180A dilutions								Normal rabbit serum controls			
	0	10	20	40	80	160	320	640	0	10	20	40	80	160	320	640	317	4984	394	180 A
H. vaginalis 4984 (King)	+	+	+	±	−	−	−	−	−	.	.	.	.	.	.	.	−	−	−	−
H. vaginalis 317 (Dukes)	+	+	+	+	±	−	−	−	−	.	.	.	.	.	.	.	−	−	−	−
H. vaginalis 394 (Dukes)	+	+	+	+	+	+	±	−	−	.	.	.	.	.	.	.	−	−	−	−
H. vaginalis 842 (U/L)	+	+	+	±	−	−	−	−	−	.	.	.	.	.	.	.	−	−	−	−
H. vaginalis T 145 (Edmunds)	+	+	+	±	−	−	−	−	−	.	.	.	.	.	.	.	−	−	−	−
H. aegyptius 180 A (Pittman)	−	.	.	.	.	.	.	.	+	+	+	+	+	±	−	−	−	−	−	−

* Fluorescein-tagged *H. vaginalis* 4984 antiserum was used in the inhibition reactions with non-fluorescent antiserum 394.
+, complete inhibition (no fluorescence); ±, incomplete inhibition (weak fluorescence); −, no inhibition (fluorescence).

size of basic fuchsin-stained organisms ranged from 0·4–0·6 $\mu \times 1{\cdot}0$–2·0 μ for the Dukes, King, Edmunds and U/L strains, whereas the Amies strains tended to be slightly longer, 2·0–3·5 μ.

Organisms from such cultures were Gram-negative. Gram variability was noted occasionally with some strains (Edmunds, King) in smears made from 48 and 72 hr. In Casman rabbit blood broth cultures, such Gram variability was not observed with the Amies (H 2030), Dukes or U/L (842) strains. Bipolar staining was observed in some Giemsa stained organisms from Casman rabbit blood agar cultures of Dukes, Edmunds, King and U/L strains. Two of the Amies strains (H 36, H 76) did not show bipolar staining, while strain H 2030 showed a few bipolar organisms. The Amies strains showed filamentous forms on Casman rabbit blood agar.

Table 4. *Direct immunofluorescent reaction*

	Fluorescent antiserum		
Organism and source	*H. vaginalis* 317	*H. vaginalis* 4984	*H. aegyptius* 180 A
H. vaginalis 317 (Dukes)	+	+	−
H. vaginalis 394 (Dukes)	+	+	−
H. vaginalis 4984 (King)	+	+	−
H. vaginalis T 104 (Edmunds)	+	+	−
H. vaginalis T 145 (Edmunds)	+	+	−
H. vaginalis 680 (U/L)	+	+	−
H. vaginalis 842 (U/L)	+	+	−
H. vaginalis 867 (U/L)	+	+	−
H. vaginalis H 36 (Amies)	−	−	+
H. vaginalis H 76 (Amies)	−	−	±
H. vaginalis H 2030 (Amies)	−	−	+
H. aegyptius 180 A (Pittman)	−	−	+
H. aegyptius 181 A (Pittman)	−	−	+
H. influenzae 9006 (ATCC)	−	−	+
H. influenzae 9007 (ATCC)	−	−	+
H. influenzae 9008 (ATCC)	−	−	+
H. influenzae 9334 (ATCC)	−	−	+
H. influenzae 8142 (ATCC)	−	−	+

+, fluorescence; ±, weak fluorescence; −, no fluorescence.

Colonial morphology. The colonial morphology of the various *Haemophilus vaginalis* strains on Casman rabbit blood agar were studied after 24 hr. and 48 hr. incubation in a candle jar. The Dukes, Edmunds, King and U/L strains formed colonies like those described by Dukes & Gardner (1961). The Amies strains, however, were umbonate, entire, translucent and slightly granular, varying in size from 0·37–0·97 mm. in diameter. They were similar in this respect to *H. aegyptius* strain 180 A.

Haemolytic activity on rabbit red cells in Casman medium showed a wide range of variability from no reaction to slight green colour, and to greenish-brown after 24 hr. for all strains of *H. vaginalis*; a greenish area with haemolysis was most frequently observed, however. No haemolysis was produced by *H. aegyptius*.

Maintenance on various media. The maintenance of *Haemophilus vaginalis* strains and one strain of *H. aegyptius* was studied. The results of these studies are summarized in Table 1 from which it is apparent that whole blood or perhaps serum is necessary for the maintenance of Dukes, Edmunds, King and U/L strains.

Table 5. *Indirect immunofluorescent reaction*

Antigen	Non-fluorescent *H. vaginalis* antiserum 317 dilutions								Non-fluorescent *H. vaginalis* antiserum 4984 dilutions							
	0	10	20	40	80	160	320	640	0	10	20	40	80	160	320	640
H. vaginalis 4984 (King)	4	4	3	1	1	—	.	.	4	4	4	3	2	2	1	—
H. vaginalis 317 (Dukes)	4	4	4	4	3	2	1	—	4	2	1	1	1	—	.	.
H. vaginalis 394 (Dukes)	4	2	1	—	.	.	.	.	4	2	1	—	.	.	.	.
H. vaginalis 842 (U/L)	3	2	1	—	.	.	.	.	4	2	1	tr	—	.	.	.
H. vaginalis T 145 (Edmunds)	4	3	2	1	tr	—	.	.	4	4	3	2	1	1	—	.
H. vaginalis H 2030 (Amies)	—	.	.	.	.	.	.	.	—	.	.	.	.	.	.	.
H. vaginalis H 76 (Amies)	—	.	.	.	.	.	.	.	—	.	.	.	.	.	.	.
H. vaginalis H 36 (Amies)	—	.	.	.	.	.	.	.	—	.	.	.	.	.	.	.
H. aegyptius 180A (Pittman)	—	.	.	.	.	.	.	.	—	.	.	.	.	.	.	.

Table 5 (*cont.*)

Antigen	Non-fluorescent *H. vaginalis* antiserum 394 dilutions								Non-fluorescent *H. aegyptius* antiserum 180A dilutions								Normal rabbit serum control			
	0	10	20	40	80	160	320	640	0	10	20	40	80	160	320	640	317	4984	394	180A
H. vaginalis 4984 (King)	4	2	1	tr	—	.	.	.	—	.	.	.	.	.	.	.	—	—	—	—
H. vaginalis 317 (Dukes)	4	2	1	1	tr	—	.	.	—	.	.	.	.	.	.	.	—	—	—	—
H. vaginalis 394 (Dukes)	4	4	3	3	2	1	—	.	—	.	.	.	.	.	.	.	—	—	—	—
H. vaginalis 842 (U/L)	4	2	1	—	.	.	.	.	—	.	.	.	.	.	.	.	—	—	—	—
H. vaginalis T145 (Edmunds)	4	3	2	1	—	.	.	.	—	.	.	.	.	.	.	.	—	—	—	—
H. vaginalis H 2030 (Amies)	—	.	.	.	.	.	.	.	3	2	1	—	.	.	.	.	—	—	—	—
H. vaginalis H 76 (Amies)	—	.	.	.	.	.	.	.	3	2	1	1	—	.	.	.	—	—	—	—
H. vaginalis H 36 (Amies)	—	.	.	.	.	.	.	.	3	2	1	—	.	.	.	.	—	—	—	—
H. aegyptius 180A (Pittman)	—	.	.	.	.	.	.	.	4	4	3	3	2	tr	—	.	—	—	—	—

4 to 1, degree of fluorescence observed (4 = maximum); tr, weak fluorescence observed; —, no fluorescence observed.

Agglutination tests. Results of tube agglutination reactions at 3 hr. and 18 hr. incubation are shown in Table 2. The highest agglutination titres were obtained in homologous antigen and antiserum reactions. Significantly high titres were obtained, however, in some of the heterologous reactions with *Haemophilus vaginalis*. The titres of the Amies strains (H 76 and H 2030) with the antisera of *H. vaginalis* 317, 4984 and 394 are not significant, although the titres for the *H. aegyptius* 180 A antiserum may be significant. Spontaneous agglutination of *H. vaginalis* H 36 (Amies) was not avoided even when 0·1 % bovine serum albumin modified Hanks balanced salt solution was used; therefore, this strain was not included in the tube agglutination studies.

Immunofluorescent reactions. Specificity of the fluorescent *Haemophilus vaginalis* (317 and 4984) and *H. aegyptius* (180 A) antisera were determined by the inhibition reactions (see Table 3). Inhibition by non-fluorescent antiserum was greatest in the homologous antigen-globulin reaction. However, the non-fluorescent *H. vaginalis* antiserum reacted with heterologous *H. vaginalis* strains, resulting in inhibition of the fluorescent reaction. No significant inhibition was noted when non-fluorescent *H. vaginalis* 317 and 4984 antisera were mixed with *H. aegyptius* 180 A antigen.

The results of the cross-reaction studies by the direct method with species of Haemophilus are shown in Table 4. *H. vaginalis* fluorescent globulins 317 and 4984 reacted with homologous and heterologous *H. vaginalis* strains Dukes, Edmunds, King and U/L, but not with the Amies strains (H 36, H 76, H 2030). These Amies strains did show a reaction with *H. aegyptius* fluorescent globulin 180 A, as did *H. influenzae* strains and the *H. aegyptius* strains. Strains of *H. vaginalis* (Dukes, King and U/L) other than those listed in Table 4 were also studied; all of these showed a fluorescent reaction of varying intensity with *H. vaginalis* fluorescent globulins 317 and 4984. No fluorescent reaction was observed with these strains when *H. aegyptius* fluorescent globulin 180 A was used. Indirect immunofluorescent reactions were made to further substantiate the specificity of direct and inhibition tests. The results are in Table 5.

DISCUSSION

The morphological and colonial characteristics of the Edmunds, King and U/L strains of *Haemophilus vaginalis* grown on Casman rabbit blood agar were consistent with the report of Dukes & Gardner (1961). The characteristics of the Amies strains, however, differed on this medium, where they formed larger and umbonate colonies. Gram variability was noted in only two strains (Edmunds and King), and was not a consistent phenomenon in these two strains. In agreement with Dukes & Gardner (1961), bipolar staining was observed frequently in Dukes, Edmunds, King and U/L strains of *H. vaginalis*, but was observed in only one Amies strain (H 2030).

All investigators with the exception of Amies & Jones (1957) have noted that whole blood from one or another animal was a cultural requirement for strains of *Haemophilus vaginalis*. In the present work it was noted that Amies strains could be easily maintained on peptic digest agar and chocolate-yeastolate agar, both of which contain factors derived from whole blood. However, the Dukes, Edmunds, King and U/L strains could not be maintained on these media, thus indicating that

some factor in whole blood or serum was needed by the latter strains, and was not provided by the whole blood derivatives.

Lapage (1961) used Casman agar in addition to other media in comparing his isolates with strains of *Haemophilus vaginalis* from Drs C. R. Amies and C. D. Dukes. Lapage established two cultural groups: (1) *H. influenzae*-like, which included the Amies strains of *H. vaginalis*; (2) *H. vaginalis*-like, which included a Dukes strain of this organism. On Casman rabbit blood agar a similar grouping was found in the present work, the Amies strains being similar to *H. aegyptius* in maintenance and morphology, while the Edmunds, King and U/L strains were identical with the strains of *H. vaginalis* obtained from C. D. Dukes.

In the present work, the majority of strains produced a haemolysis as described by Dukes & Gardner (1961); however, an occasional strain produced no haemolysis or produced an inner zone of β-haemolysis, in agreement with Edmunds (1960*a*). Because of these varied results, we believe that haemolysis is of little value in the identification of *Haemophilus vaginalis* colonies on Casman rabbit blood agar.

Colonies of Lactobacillus, Streptococcus and diphtheroids may be easily confused with *Haemophilus vaginalis* colonies macroscopically; it is therefore important to examine the minute colonies microscopically for granularity and irregular shape.

Haemophilus vaginalis is an extremely fastidious organism, and maintenance of stock cultures over long periods of time was difficult. Best results were obtained when the Dukes, Edmunds, King and U/L strains were transferred daily in Casman rabbit blood broth; other media suggested for the maintenance of the genus *Haemophilus* failed to support their growth. Casman rabbit blood broth and agar were used as the medium of choice for *H. vaginalis*. Amies strains differed from the above strains in that they could not be maintained in Casman rabbit blood broth; they were maintained successfully by transferring at 3- to 5-day intervals on chocolate-yeastolate agar or on Casman rabbit blood agar.

Dukes & Gardner (1961) stated that the X and V factors are stimulatory accessory growth factors for *Haemophilus vaginalis* in the presence of partially digested protein, rabbit serum and certain amino acids. We found it impossible to supply the nutritional requirements of these strains by using a peptic digest prepared from whole rabbit blood or by using yeastolate with whole rabbit blood that had been denatured by heat. Thus, some factor or factors other than the X and V of whole blood are necessary for these strains. On the other hand, X and/or V factor requirements of Amies strains could be supplied by derivatives of whole rabbit blood such as a peptic digest of such blood or chocolate-yeastolate agar.

Agglutination reactions. The addition of 0·1 % bovine serum albumin to a modified balanced salt solution (e.g. Hanks) eliminated spontaneous agglutination in tube reactions with all strains of *Haemophilus vaginalis* except Amies strain H36. No cross-reactions occurred with *H. influenzae*, *H. aegyptius* and Amies strains of *H. vaginalis* and the three *H. vaginalis* antisera (317, 394 and 4984). Homologous and heterologous strains of *H. vaginalis* (Edmunds, Dukes, King and U/L) were agglutinated by the three *H. vaginalis* antisera. In this investigation as in the investigation of Dukes & Gardner (1961) the homologous strains produced high titres, whereas the heterologous strains produced lower titres.

Amies & Jones (1957) did not observe cross-reactions with antisera for their strains of *Haemophilus vaginalis* and strains of *H. influenzae* and *Bordetella pertussis*

antigens by the slide-agglutination test; however, they did not include *H. aegyptius* in their investigation. *H. aegyptius* antiserum agglutinated Amies strains of *H. vaginalis* at titres of 1/80 and did not agglutinate Dukes, Edmunds, King and U/L strains of *H. vaginalis* or *H. influenzae*. Pittman & Davis (1950) stated that cross-reactions with heterologous strains of *H. aegyptius* and *H. aegyptius* antisera at titres of 1/40 were significant. In view of the cross-reactions observed with Amies strains of *H. vaginalis* and *H. aegyptius* antiserum in the present work, it appears that these strains are more closely related to *H. aegyptius* than to *H. vaginalis*.

Immunofluorescent reactions correlated with the agglutination reactions. Cross-reactions did not occur in reactions with *Haemophilus vaginalis* antiserum (317 and 4984) and antigens (whole bacteria) of *H. influenzae*, *H. aegyptius*, and Amies strains of *H. vaginalis*. Reactions did occur, however, with *H. vaginalis* antiserum (317 and 4984) and any of the Dukes, Edmunds, King and U/L strains of *H. vaginalis*. On the basis of these observations, it appears there are no common antigens shared by *H. vaginalis*, *H. influenzae* and *H. aegyptius* on the one hand and Amies strains of *H. vaginalis* on the other. Cross-reactions did occur with *H. aegyptius* fluorescent globulin and Amies strains of *H. vaginalis* and *H. influenzae* by the direct immunofluorescent method, thus indicating common antigens present in the three species. Further evidence of Amies strains of *H. vaginalis* sharing common antigens with *H. aegyptius* was provided by the indirect immunofluorescent method.

The inhibition and indirect immunofluorescent titres differed from the tube-agglutination titres with the same serum by a twofold or greater dilution factor. Such variation has been noted previously by Goldman (1957) during his investigation of Toxoplasma, and by Biegeleisen, Bradshaw & Moody (1962) in their investigations of Brucella anti-bodies in human serum. Although such titres varied, the immunofluorescent reactions are considered to be highly specific.

The specificity of the fluorescent *Haemophilus vaginalis* (317 and 4984) globulin was determined by inhibition and direct immunofluorescent methods. Cross-reactions which occurred with other species of *Haemophilus* and undiluted fluorescent globulin were avoided by diluting the fluorescent antisera 1/12 in the direct immunofluorescent method.

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Comparison of Cultural and Immunofluorescent Procedures in the Identification of *Haemophilus vaginalis*

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SUMMARY

The application of immunofluorescent techniques for the detection and identification of *Haemophilus vaginalis* in vaginal secretions by using fluorescent *H. vaginalis* antiglobulin (strains nos. 317, 4984) were found to be as specific and sensitive as cultural methods and had the advantage of being simple and rapid.

INTRODUCTION

Several investigators (Leopold, 1953; Gardner & Dukes, 1954, 1955; Gardner, Dampeer & Dukes, 1957; Amies & Jones, 1957; Brewer, Halpern & Thomas, 1957; Edmunds, 1960; Lapage, 1961) have reported the occurrence of a small, Gram-negative bacillus in the male and female genitro-urinary tract. These investigators used various media for the isolation and cultivation of this organism, named *Haemophilus vaginalis* by Gardner & Dukes (1955). Although most of the investigators used the criteria for identification established by Gardner & Dukes, several of the reports varied as to the cultural behaviour and staining characteristics. The organism was regarded as quite fastidious. In view of these problems, it was decided to compare immunofluorescent procedures, which had been found to be highly specific (Redmond & Kotcher, 1963) with cultural procedures, Gram-stained smears, and 'clue-cells' in the isolation and identification of this organism from clinical material at Houston, Texas and Louisville, Kentucky, U.S.A.

METHODS

Media. In the cultural procedures used in Louisville the following media were employed for the isolation of organisms from vaginal swabs which had been put into proteose peptone no. 3 (Difco) carrying broth; Casman agar with 5% sterile, defibrinated rabbit blood; Rogosa agar; blood agar (human out-dated blood); eosin-methylene blue agar; GC medium (Difco); PPLO broth and agar (Difco); National Institutes of Health thioglycollate with and without 5% horse serum; and Douglas broth.

In the cultural procedures used at Houston, Brewer thioglycollate broth without methylene-blue indicator (Baltimore Biological Laboratories) was used as the transport medium for clinical specimens. Casman rabbit blood agar was used for isolation. Any organism that could not be identified from this primary culture was subcultured on special media for identification. In both laboratories (Louisville, Houston) the Casman plates were cultured in a candle jar at 37°. The vaginal swabs in transport medium were streaked on cultivation media within 2–6 hr. after having

been obtained from the patient. The criteria for the identification of *Haemophilus vaginalis* were those given by Dukes & Gardner (1961).

Staining methods. The staining methods, immunization of rabbits, the method of conjugating fluorescein isothiocyanate to the globulin from the antiserum of immunized rabbits, and the immunofluorescent procedures were as described by Redmond & Kotcher (1963).

Clinical methods. Each patient was examined for symptoms of vulvitis, vaginitis and cervicitis by a gynaecologist. Vaginal secretion smears were made on three microscope slides and a vaginal swab was inoculated into a transport medium. A drop of vaginal secretion was examined by wet-mount for the presence of 'clue cells', leucocytes, *Trichomonas vaginalis*, and yeast-like organisms. One vaginal smear was Gram-stained and examined for the presence of small Gram-negative bacilli and 'clue cells'. A second vaginal smear was fixed in absolute methanol for 5 min., air dried, and stained with fluorescent *Haemophilus vaginalis* antiglobulin by the direct method. This smear was examined for fluorescent bacteria. The third vaginal smear was fixed in absolute methanol and held in reserve.

Table 1. *Comparison of four methods in studying* Haemophilus vaginalis *in three groups of patients*

Number of patients and group			Method			
27 private patients (Houston, Texas)	54 private patients (Louisville, Kentucky)	51 clinic patients (Louisville, Kentucky)	Culture	Fluorescent antibody	Clue cell*	Gram stains
14	3	5	+	+	+	+
0	0	0	+	+	+	−
0	0	0	+	+	−	−
0	0	0	+	−	−	−
3	3	1	−	−	−	−
0	1	1	−	+	+	+
0	1	2	−	−	+	+
5	36	38	−	−	−	+
0	0	0	+	−	+	+
4	5	3	+	+	−	+
0	0	0	−	+	+	−
0	0	0	−	+	−	−
0	1	0	−	−	+	−
0	0	1	+	−	−	+
1	4	0	−	+	−	+
0	0	0	+	−	+	−

* Clinic patients were examined for clue cells by Gram-stained smears rather than by wet mount.

RESULTS

A comparison of four methods for studying *Haemophilus vaginalis*, namely, culture, FA staining, presence of 'clue cells', and Gram reaction is presented in Table 1. FA staining revealed the presence of *H. vaginalis* in five patients from whom the organism was not cultured. In no instance was the organism cultured and not found on vaginal smears by FA staining. The 'clue cell' and the Gram-reaction observations are incorporated in Table 1 although it is realized that these techniques are not specific for *H. vaginalis*.

The incidence of *Haemophilus vaginalis*, as revealed by FA staining, accepted cultural procedures and by both methods in the same three groups of patients is presented in Table 2. The Houston patients were highly selected because it was decided to use such a group as a control to determine the sensitivity and specificity of the methods. No such selection was carried out with either group at Louisville.

Table 2. *Incidence of* Haemophilus vaginalis *by fluorescent antibody and cultural methods*

Number of patients and group	Positive culture		Positive fluorescent antibody		Positive culture and fluorescent antibody	
	No.	%	No.	%	No.	%
27 private patients, Houston, Texas	19	70	19	70	19	70
54 private patients, Louisville, Kentucky	8	15	13	24	8	15
51 clinic patients, Louisville, Kentucky	9	18	9	18	8	16

A great variety of organisms may be found in the vagina, particularly in patients with vaginitis, cervicitis and vulvitis. In Table 3 a list is given of other organisms found in association with *Haemophilus vaginalis* in the three groups of patients examined. There was a considerable difference in the frequency with which certain organisms, such as *Staphylococcus aureus*, *Streptococcus viridans*, *Micrococcus* sp., *Escherichia coli*, diphtheroids, and Doederlein's bacillus were isolated as between the Houston patients and the Louisville patients. However, different cultural procedures were used and different bacteriologists were involved in studying the patients of the two cities.

Table 3. *Other organisms found in association with* Haemophilus vaginalis *in vaginal secretions*

Organisms associated with *Haemophilus vaginalis*	Number of patients and group		
	19 private patients (Houston, Texas)	13 private patients (Louisville, Kentucky)	10 clinic patients (Louisville, Kentucky)
Trichomonas vaginalis	3	2	3
Candida sp.	1	1	0
Staphylococcus aureus	1	5	5
Streptococcus viridans	8	1	0
S. anhemolyticus	0	1	0
S. hemolyticus	0	2	0
Enterococcus	0	2	0
Sarcina sp.	0	3	1
Micrococcus sp.	6	0	0
Escherichia coli	0	3	1
Diphtheroids	16	1	4
Doederlein's bacillus	0	10	4
No organism	1	0	2

The complete flora studies on the Louisville, Kentucky, groups were done by Miss Carolyn Frick. The flora studies on the Houston, Texas group were done by Mrs Martha Foster.

DISCUSSION

In interpreting the results of the three patients groups, the Houston group was considered as the control group. The clinician selected the patients by clinical signs and symptoms of *Haemophilus vaginalis* vaginitis, and also included normal women, and women with vaginitis caused by organisms other than *H. vaginalis* in this group. The two groups of patients from Louisville were randomly selected patients; however, some patients from the private practice group were suspected by clinical observation of having *H. vaginalis* vaginitis.

The fluorescent *Haemophilus vaginalis* antiglobulin was as sensitive as cultural techniques in identifying *H. vaginalis*. In the clinical specimens examined, FA methods revealed six additional specimens (five patients) with *H. vaginalis* which was not isolated by cultural methods. Non-specific fluorescence was ruled out by subjecting the other species of bacteria isolated to staining with fluorescent *H. vaginalis* antiglobulin by the direct immunofluorescent method. In a single case from the Louisville gynaecology clinic, *H. vaginalis* was isolated by cultural methods, whereas the vaginal smear from this patient was negative by direct immunofluorescent reaction. However, the culture of *H. vaginalis* was positive for fluorescence by the direct immunofluorescent reaction.

Because of the fastidious nature of *Haemophilus vaginalis*, it is necessary to use a highly enriched culture medium, such as Casman rabbit blood agar, for the isolation of the organism. On this medium other organisms from the vagina grow rapidly and may, on occasion, overgrow *H. vaginalis*. In cultivating this fastidious bacillus from clinical specimens it is important that the specimens do not remain in transport media more than 6–8 hr. or the organisms will not survive. Because of these cultural and transport difficulties, it is believed that FA techniques are more practical in the identification of *H. vaginalis* particularly as in this investigation the use of fluorescent *H. vaginalis* antiglobulin was as specific and somewhat more sensitive than culture methods. Also, it has the advantage of simplicity and rapidity, as well as not requiring the processing of the clinical specimen immediately, which is necessary with cultural procedures.

The difference in the percentage incidence of *Haemophilus vaginalis* in the Houston patients as compared with the Louisville patients is explained by the fact that the Houston gynaecologist selected his patients for *H. vaginalis* vaginitis. The two Louisville groups were unselected, and the incidence in this series of patients was comparable to that found by Gardner *et al.* (1957), Ritzerfeld & Kummel (1960), Kummel & Ritzerfeld (1961), and Wurch & Lutz (1955). The incidence of *H. vaginalis* in Louisville, however, was lower than that reported by Brewer *et al.* (1957), Edmunds (1959) and De La Fuente, Rico & Soria (1959).

Although Gardner & Dukes (1955) recommended the wet mount for the detection of 'clue cells', other investigators (Edmunds, 1959; Lapage, 1961) have also used the Gram-stained vaginal smear. In the present work, patients from the Louisville clinic group were examined for 'clue cells' by Gram-stained smears. Small Gram-positive organisms were frequently found attached to the epithelial cells, thus producing the same phenomenon in wet mounts as when *Haemophilus vaginalis* was attached to the cells. In the two Louisville groups, 'clue cells' were present when cultural and immunofluorescent methods did not reveal *H. vaginalis* (Table 1);

it was also noted that *H. vaginalis* was found when 'clue cells' were absent. LaPage (1961) observed Gram-positive and Gram-negative mixed 'clue cells', thus casting doubt on the significance of the association of such cells with the isolation of the small Gram-negative bacillus.

Comparison of the organisms found in association with *Haemophilus vaginalis* in the vaginal secretions of the three groups studied showed a marked difference as between the Houston group and the two Louisville groups (Table 3). The flora studies of the three groups were not made with identical transport and cultural media, nor were the bacteriologists the same. These technical differences are probably the most satisfactory explanation for the differences observed in the vaginal floras. The absence of Doederleins' bacillus from the Houston group and its presence in the two Louisville groups is the most striking feature. However, Rogosa medium or tomato juice agar medium was not used in studying the Houston group, and for this reason Doederlein's bacillus may have been missed.

In comparing the two Louisville groups, Doederlein's bacillus and *Staphylococcus aureus* were the organisms most frequently associated with *Haemophilus vaginalis*. *Trichomonas vaginalis* was associated with *H. vaginalis* in 18 % of the Louisville private practice group, 28 % of the Louisville clinic group, and 16 % of the Houston private practice group. Diphtheroids were frequently isolated from the Louisville clinic group and the Houston private practice group; however, they are considered to be part of the normal vaginal flora. Brewer *et al.* (1957), Heltai (1959), Edmunds (1959), and LaPage (1961) stated that *H. vaginalis* was isolated more frequently with a mixed microbial flora. In the present work too, more often than not, *H. vaginalis* was isolated together with a mixed flora. The frequently observed pure cultures of *H. vaginalis* reported by Gardner & Dukes (1955) were noted in only three instances out of 122 patients in the present work.

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Isolation of N-Acetylneuraminic Acid and 4-Oxo-norleucine from a Polysaccharide Obtained from *Citrobacter freundii*

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SUMMARY

A polysaccharide constituted primarily of a unit of *N*-acetylneuraminic acid, two of glucosamine and one of an acid labile nitrogenous component was extracted from *Citrobacter freundii* O5:H30. The material after purification gave a single peak in the ultracentrifuge. *N*-acetylneuraminic acid and 4-oxo-norleucine were isolated from hydrolysates of the polysaccharide. A similarly constituted polysaccharide was obtained from *Salmonella dahlem*. *C. freundii* O5:H30 and *S. dahlem* were shown previously to be serologically related to one another and the results of the present investigations indicate that a chemical relationship also exists between these micro-organisms. Moreover, it is suggestive that the serologically related *S. djkarta* which contains neuraminic acid is related chemically to *S. dahlem* and *C. freundii* O5:H30. In sum, it is concluded that derivatives of neuraminic acid exist in association with other amino sugars and amino acids in mucopolysaccharides of bacterial origin as well as in those derived from mammalian origin.

INTRODUCTION

Mucopolysaccharide complexes obtained from excretions or from extracts of animal tissues are constituted of nitrogenous carbohydrate and of amino acids firmly bound together. The monosaccharides, glucosamine, galactosamine or their *N*-acetylated derivatives are often present. In addition, many mucopolysaccharides contain derivatives of neuraminic acid (Blix, 1936). The amino acids associated with mucopolysaccharide complexes are of the types commonly found in proteins of animal tissues.

Mucopolysaccharide complexes isolated from microbial sources are constituted of monosaccharides and of amino acids similar to those found in mucopolysaccharides obtained from higher organisms. However, amino sugars and amino acids not yet shown to be associated with materials of animal origin may also be present. These include muramic acid (3-0-α-carboxyethyl glucosamine) (Strange & Powell, 1954), D-fucosamine (2-amino-2,6-dideoxy-D-galactose) (Crumpton & Davies, 1958), diaminohexose (Sharon & Jeanloz, 1959), mannosamine (Rude & Goebel, 1962), and more recently a 4-amino-4,6-dideoxy-aldohexose (Wheat, Rollins & Leatherwood, 1962) among the amino sugars. α,ε-Diamino-pimelic acid (Work, 1951) and many D-isomers of the common amino acids such as D-alanine, D-glutamic and D-aspartic have also been found. A review which summarizes the numerous components present in complex mucopolysaccharides derived from bacteria has been published by Work (1961).

Derivatives of neuraminic acid have rarely been detected in products derived from bacteria. Up to the present time only two polysaccharides which contain these materials have been obtained from micro-organisms. Colominic acid, a polymer of *N*-acetylneuraminic acid, was isolated from culture supernatants of *Escherichia coli* which have a K1 serotype (Barry, 1958; Barry, Abbott & Tsai, 1962). A polysaccharide containing a derivative of neuraminic acid was later obtained from strains of group C *Neisseria meningitidis* (Watson, Marinetti & Scherp, 1958). Despite observations made from chemical tests that derivatives of neuraminic acid are present in other bacteria (Barry, 1959; Barry, Tsai & Chen, 1960), they have been isolated and characterized only from hydrolysates of complex substances derived from *E. coli* (Barry, 1957, 1958). Furthermore, little is known concerning the specific nature of the chemical binding of the neuraminic acids to other sugars or to amino acids in the various bacteria which contain these substances.

From the work which is reported here, it will be seen that a polysaccharide isolated from a strain of *Citrobacter freundii* contains a large amount of *N*-acetylneuraminic acid in association with glucosamine and other nitrogenous constituents. Crystalline *N*-acetylneuraminic and 4-oxo-norleucine were isolated from hydrolysates of the purified polysaccharide. Norleucine has not previously been found in natural products. However, a derivative, 6-diazo-5-oxo-L-norleucine has been obtained from a Streptomyces (Dion *et al.* 1956). Thus, a new and unique nitrogenous polysaccharide has been discovered in products of microbial origin.

METHODS

Organisms. In the following experiments several strains of micro-organisms were employed. A strain originally known as *Escherichia coli* 5396/38, which produces Vi antigen, obtained from Walter Reed Army Medical School, Washington, D.C., was kindly provided by Dr M. Landy and Dr M. Webster. Subsequent serological studies revealed this strain to be a *Citrobacter freundii* with O and H antigens related to O5 and H30 antigens present in the Arizona group of enterobacteriaceae. *Salmonella dahlem* and *S. djkarta* were kindly provided by Dr W. H. Ewing, United States Public Health Service, Chamblee, Georgia.

Media and cultivation of organisms. All strains were maintained on nutrient agar slopes incubated for 16 hr. at 37°. Larger numbers of cells were grown at 37° in a 1 % (w/v) dialysed technical casamino acid medium (Difco), 0·1 M-phosphate buffer at pH 7·0 and glucose 1·0 % (w/v) contained in 2 l. flasks or in 5 or 12 gal. pyrex glass bottles.

The apparatus employed for the mass cultivation of bacteria at fixed pH was a revision of that previously described (Goebel, Barry & Shedlovsky, 1956). A commercially available automatic titrator (Radiometer, Model TTT–1) with standard accessories, magnetic relay (MNR1) and adaptor (SGB/2) for Beckman calomel and glass electrodes was used to measure and maintain the pH of growing cultures. In addition, a stainless steel solenoid valve attached to an alkali reservoir was substituted for the solenoid operated buret (Longsworth & MacInnis, 1935). Aeration of cultures was accomplished with aquarium pumps (Marco) which deliver air at the rate of 1 l./min. each.

Chemical analyses. Nitrogen determinations were performed by the method of

Koch & McMeekin (1924); phosphorus was estimated by the procedure of Allen (1940). Protein was determined by a modified method of Folin & Ciocalteu (Kunkel & Tiselius, 1951), employing crystalline bovine albumin (Armour) as a standard. Carbohydrate was estimated by a modified anthrone procedure (Goebel & Barry, 1958) employing glucose as a standard, and hexosamine was estimated by the method of Sorensen (1938). Hexuronic acids, heptoses and pentoses were ascertained by the procedures of Dische (1947) and Dische, Shettles & Osnos (1949). Neuraminic acid estimations were performed by the method of Warren (1959) and by a modified Ehrlich procedure (Barry *et al.* 1962). Amino acids were analysed by the ninhydrin procedure of Moore & Stein (1954). Content of nucleic acid was calculated from the absorbance obtained at 260 mμ, using yeast ribonucleic acid as a standard. Lipid was determined by weighing ether soluble material liberated after hydrolysis for 1 hr. at 100° with 1 N-hydrochloric acid.

Elemental analyses were performed by Galbraith Laboratories, Inc., Knoxville, Tennessee. Acetyl determinations were also made by this laboratory using the procedure of Clark (1936).

Elemental analyses were also performed by Schwarzkopf Microanalytical Laboratory, New York, N.Y. Amino nitrogen determinations by the method of Van Slyke were made by this laboratory.

Partition chromatographic analysis of *N*-acetylneuraminic acid and derivatives was performed at 24° by the descending method on Whatman no. 1 filter paper, in the following solvent systems: (*a*) 80 g. phenol:20 g. water:0·04 g. 8-hydroxyquinoline in ammonia saturated atmosphere; (*b*) 2,3-lutidine + water (6·5 + 3.5 v/v); (*c*) butan-1-ol + pyridine + water (3 + 2 + 1·5, v/v). The mobility of *N*-acetylneuraminic acid is 0·20, 0·30 and 0·16 relative to the solvent front in systems *a*, *b* and *c*, respectively. *N*-glycolylneuraminic acid *N*-4-*O*-diacetylneuraminic acid, *N*-7-O-diacetylneuraminic acid can be separated from *N*-acetylneuraminic acid in one or more of these systems. The procedures of Warren (1960) and of Barry (1958) were employed to stain the chromatograms.

Partition chromatographic analysis of amino acids and of amino sugars was performed at 24° on sheets of Whatman no. 1 filter paper. A 6 N-hydrochloric acid hydrolysate of the material to be analysed was prepared by the method of Craig, Weisiger, Hausmann & Harfenist (1952). Two-dimensional paper chromatograms were run. The solvent system butan-1-ol + acetic acid + water (4 + 1 + 5, v/v) was first used to develop the chromatogram by the descending technique for 16 hr. After drying, the paper was rotated 90°, rolled into a cylinder and placed into a jar. The chromatogram was now developed by the ascending technique for 4 hr. using pyridine + water (4 + 1, v/v) as the solvent. The chromatograms after drying were sprayed with 0·4 % ninhydrin in acetone or with a ninhydrin reagent composed of 5 ml. of 0·5 % ninhydrin (w/v) in 95 % alcohol + 20 ml. of 95 % ethanol + 8·5 ml. glacial acetic acid + 3·5 ml. of γ-collidine and heated at 100° for 3 min.

Partition chromatography to detect mannosamine and glucosamine was performed on borate treated paper by the method of Cardini & Leloir (1957).

Physical analyses. Ultracentrifuge analyses were performed in a Spinco Model E analytical centrifuge at 59,780 rev./min. in sodium borate pH 9·2 and in sodium acetate pH 4·5 buffers of 0·1 ionic strength.

Biological analyses. Antisera were obtained from rabbits which had received

multiple intravenous injections of formalized bacteria followed by inoculations with viable cells in accordance with the procedures described by Edwards (1951) and Edwards & Ewing (1955) for enteric micro-organisms.

Agglutination tests were conducted in the usual manner employing 18 hr. nutrient broth cultures. Precipitin tests were performed in the manner described by Barry *et al.* (1962).

Chemical methods. Hydrogenation experiments were performed as follows: into a 25 ml. hydrogenation flask equipped with a magnetic stirrer was placed 20 mg. platinum oxide (Adams catalyst) and 5 ml. water. The system was evacuated and filled with hydrogen gas. The process of evacuation and filling with hydrogen gas was repeated several times to insure complete removal of oxygen. The system was finally filled with the gas. The suspension was now stirred until the brownish catalyst turned completely black and no more hydrogen was adsorbed. The stirrer was stopped and a solution containing 10–20 mg. material dissolved in 5 ml. water was slowly added to the flask through the funnel. The stopcock was closed prior to the addition of the final 0·2 ml. and 5 ml. water added to rinse the funnel. This solution was also permitted to drain into the flask until the final 0·2 ml. The washing was repeated with another 5 ml. water. The suspension was now stirred for 150 min. at 25° and the volume of gas adsorbed measured.

Ion exchange resins employed for column chromatography were prepared as follows: amberlite IRC 50 resin (Rohm & Haas) was purchased in the H^+ form. Columns were prepared by thoroughly washing with 0·2 M-ammonium acetate buffer at pH 6·1 followed by distilled water.

Dowex 1-X8 resin (Biorad Lab) was purchased in the Cl^- form. Columns were prepared by washing with 2 N-sodium hydroxide until free of chloride ion. This was followed by washing with distilled water and 2 N-formic acid. The column was next washed with 2 M-ammonium formate at pH 7·8 followed by a rinse with distilled water.

Dowex AG 50W-X4 resin (Biorad) was purchased in the H^+ form. Columns were prepared by washing with 6 N-hydrochloric acid followed by distilled water until the pH was near neutrality.

Chemical preparations. The group C *Neisseria meningitidis* hapten employed was kindly provided by Dr G. Watson, Bowman Gray Medical School, Winston-Salem, North Carolina. Preparations of Vi antigen were generously supplied by Dr E. E. Baker, Boston, Massachusetts, and by Dr M. Webster. The 3-hydroxy-norleucine was kindly supplied by Dr T. T. Otani, National Institutes of Health, Bethesda, Maryland. Colominic acid was prepared in this laboratory from culture supernatants of *Escherichia coli* O1:K1:HNM, O2:K1:HNM or O7:K1:HNM.

Preparation of Citrobacter freundii *polysaccharide.* Thirty litres of culture medium was seeded with 2 ml. of a 10^{-5} broth dilution of *Citrobacter freundii* O5:H30 growing in the logarithmic phase. The culture was maintained at 37° at pH 7·0 and aerated at the rate of 2 l./min. When growth of the organisms ceased, 100 ml. chloroform was added and aeration continued for 20 min. The bacteria were separated in a Sharples centrifuge and washed by suspension in 1 l. water and collected by centrifugation. The cells were finally resuspended in 300 ml. water and lyophilized. A yield of 40–50 g. of dried bacilli was usually obtained.

191 g. dried cells, containing 1·4 % *N*-acetylneuraminic acid, isolated from four

separate 30 l. cultures was suspended in 1 l. of acetone and incubated for 16 hr. at 37°. The suspension was filtered through a Buchner funnel and the filtrate discarded. The air-dried residue was suspended in 8 l. water and autoclaved for 75 min. at 121°. The mixture was clarified in a Sharples centrifuge and the residue resuspended in 2 l. water. The suspension was autoclaved for 75 min. at 121° followed by centrifugation. The residue was discarded and the combined supernatants concentrated *in vacuo* (1–2 mm.) at 20° in a modified glass-circulating evaporator to 1·5 l. (Barry & Pierce, 1959). The solution was dialysed in 18/32 sausage casings against 15 l. distilled water. The bag contents were concentrated *in vacuo* to 800 ml. dialysed and concentrated to 500 ml. The final concentrate, after lyophilization, gave 68 g. of substance containing 3·5 % of *N*-acetylneuraminic acid. The material was extracted with 2 l. of 0·02 M-sodium acetate buffer at pH 4·0 and centrifuged. The supernatant was decanted, concentrated *in vacuo* to 200 ml., dialysed and lyophilized.

Further purification was accomplished by precipitating an aqueous solution of the crude material with ethanol at −10°. To a 3 % solution of the crude material dissolved in 0·05 M-sodium acetate buffer at pH 5·6 and cooled to 0° was added absolute ethanol (−10°) to a concentration of 50 %. The solution was kept at −20° in a deep freeze for 1 hr. followed by centrifugation. The precipitate (fraction 1) was dissolved in 100 ml. water, dialysed, concentrated *in vacuo* and lyophilized. To the supernatant was added additional cold ethanol to a concentration of 75 % and the mixture was stored at −20° for 1 hr. The precipitate (fraction 2) was collected by centrifugation. It was dissolved in 75 ml. water, dialysed, concentrated *in vacuo* and lyophilized. The alcohol supernatant (fraction 3) was dialysed, concentrated *in vacuo* to 50 ml. and lyophilized.

Isolation of the final product was achieved by precipitating an aqueous solution of fraction 2 with ammonium sulphate at room temperature. Thus, to a 3 % solution of the material was added solid ammonium sulphate and fractions collected by centrifugation at 0·5, 0·6, 0·7, 0·8 and 0·9 saturation. The precipitates (fractions A, B, C, D and E) were separately dissolved in 50 ml. water. The final supernatant (fraction F) and redissolved fractions were dialysed against water until free of sulphate ion, concentrated *in vacuo* and lyophilized.

The removal of ribonucleic acid and of protein was accomplished by digestion of fraction D with ribonuclease followed by digestion with trypsin. To 900 mg. material, dissolved in 50 ml. of 0·1 M-sodium acetate buffer at pH 5·0 was added 50 ml. solution containing 20 μg./ml. ribonuclease (Worthington) dissolved in buffer. The mixture was dialysed at 37° against 1 l. of the buffer containing 1 ml. chloroform. The buffer was changed after each 12 hr. of incubation. Samples of the dialysate were taken at various time intervals and the absorbance measured at 260 mμ. The increase in absorbance was initially rapid but showed little change in samples taken after 24 hr. When the absorbance ceased to rise, the digest was dialysed against several changes of water and concentrated *in vacuo* to 25 ml. Twenty-five ml. solution containing 10 mg. trypsin (Worthington) dissolved in 0·15 M-phosphate buffer at pH 7·6 was added. The mixture was dialysed against 500 ml. buffer containing chloroform and incubated at 37° with a change of buffer after each 12 hr. interval. Absorbance measurements were made at 280 mμ. on samples of dialysate taken at various time intervals. Very little increase in the

absorbancy was noted after 48 hr. When the absorbance ceased to increase the digest was dialysed against several changes of water and concentrated *in vacuo* to 20 ml. Twenty ml. of 20 % (w/v) trichloroacetic acid solution was added to precipitate the enzyme protein. The mixture was centrifuged and the precipitate discarded.

The removal of ions from the supernatant was accomplished by electrodialysis. The solution was placed into a beaker and one covered end (75 mm. membrane disks of very dense porosity, Schleicher & Schuell Co.) of each of two glass U tubes 4 cm. in diameter was dipped into the solution. The open end of each U tube was placed into separate vessels containing distilled water acidified with a few drops of formic acid. Platinum electrodes placed into the vessles were connected to a direct current power supply. The U tubes were filled with the acidified aqueous solution and prior to electrodialysis a few drops of formic acid were also added to the beaker containing the sample. A 5 mA. current flow was maintained in the system by adjustment of the voltage. During the electrodialysis the solution in the electrode vessels was gradually changed by addition of distilled water and siphoning off of an equal volume of fluid. After several hours the current fell to almost zero at a potential of 1000 V. and the electrodialysis was stopped. The electrodialysed solution was then concentrated *in vacuo* to 10 ml. and lyophilized.

RESULTS

The weight recovered and content of neuraminic acid of each fraction obtained during preparation of the *Citrobacter freundii* polysaccharide are given in Fig. 1.

Ultracentrifuge analysis of Citrobacter freundii *polysaccharide.* A 1 % solution of *Citrobacter freundii* polysaccharide in sodium borate at pH 9·2 or in sodium acetate at pH 4·5, when subjected to sedimentation analysis, showed a single peak.

Properties of Citrobacter freundii *polysaccharide.* Purified *C. freundii* polysaccharide is a fluffy white hygroscopic powder freely soluble in water to give colourless solutions of low viscosity. Aqueous solutions are acidic, pH 3·8. Humin is readily formed when the polysaccharide is heated in dilute mineral acid. When heated with Ehrlich's reagent, the material gives a red colour with an absorption maximum at 530 mμ. A reddish colour with an absorption maximum at 550 mμ. is produced when the substance is heated with Bial's orcinol reagent. Tests for nucleic acid and lipid are negative. Analysis for protein shows 0·2 % by the Folin–Ciocalteu procedure. Anthrone tests are weakly positive. Hexosamine is present. Tests for methylpentoses, pentoses, heptoses and hexuronic acids are negative. *C. freundii* polysaccharide is non-antigenic; however, it readily precipitates in the sera of rabbits which have been immunized with *C. freundii* O5:H30 from which it is derived.

Chemical analysis of Citrobacter freundii *polysaccharide.* Analyses for carbon, hydrogen, nitrogen and acetyl were performed on several preparations of purified *C. freundii* polysaccharide dried to constant weight at 80°. The lower temperature was used for drying as heating to 100° resulted in some discoloration of the material. The following are the average of several analyses of *C. freundii* polysaccharide:

Substance analysed...		C	H	N	CH_3CO
C. freundii polysaccharide	Found	42·64	6·59	5·73	17·19
$(C_{35}H_{64}N_4O_{27})n$	Calculated	43·18	6·63	5·75	17·70
$(C_{35}H_{64}N_4O_{28})n$	Calculated	42·48	6·25	5·66	17·41

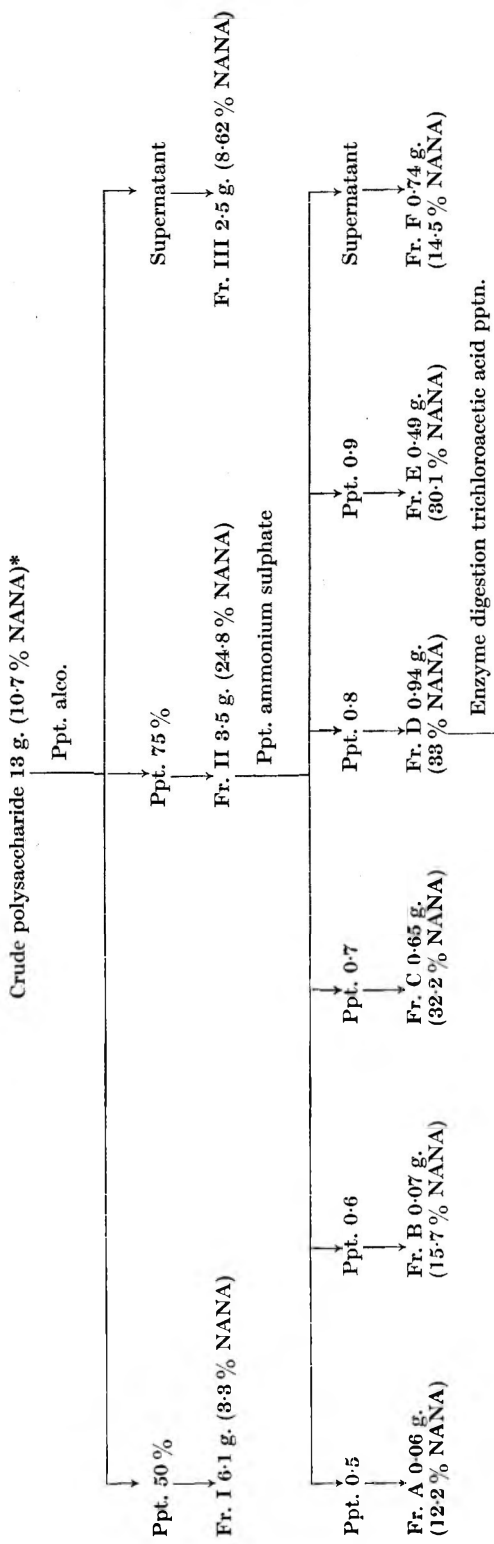

Fig. 1. Fractionation proccdure for purification of *Citrobacter freundii* polysaccharide.

From the analytical data the empirical formula $C_{35}H_{64}N_4O_{28}$ represents most closely the monomer unit of the polysaccharide. All four nitrogen atoms are believed to be acetylated. Titration of the polysaccharide with standard alkali gave a neutral equivalent of 968. The calculated value for $C_{34}H_{63}N_4O_{26}.COOH$ is 988. From this it would appear that the monomer unit of the polysaccharide contains one carboxyl group. Methoxyl groups, sulphur and phosphorus are absent. The purified material contains 34·7 % of *N*-acetylneuraminic acid and 34·8 % of glucosamine by colorimetric analyses. Both sugars were identified by partition paper chromatography. Thus, the monomer unit of *Citrobacter freundii* polysaccharide appears to be constituted of four residues: a unit of *N*-acetylneuraminic acid, two of glucosamine and an unidentified material constituted in part of one nitrogen and several carbon atoms.

The optical rotation of a 5 % aqueous solution of *Citrobacter freundii* polysaccharide, when measured in a one decimetre tube, gave the value $[\alpha]_D^{28°} = -48·5° \pm 2·0°$. An infrared spectrum of the material taken in a potassium bromide pellet using 1·4 mg. of substance mixed with 350 mg. of the salt is recorded in Fig. 4A.

Hydrolysis of Citrobacter freundii *polysaccharide.* In order to ascertain optimum conditions for hydrolysis of *C. freundii* polysaccharide which give maximum yields of free *N*-acetylneuraminic acid, a study of this reaction was made at various temperatures and pH values. As *N*-acetylneuraminic acid is readily destroyed in weak mineral acid to form humin, it was necessary to find conditions of hydrolysis which do not result in excessive destruction of the monosaccharide. The rate of liberation of *N*-acetylneuraminic acid during hydrolysis was followed by employing two different colorimetric methods of assay. Total *N*-acetylneuraminic was measured by the Ehrlich procedure and free *N*-acetylneuraminic by the Warren method. It was observed that optimum results were obtained by heating a 1 % solution of the material in 1 N-sulphuric acid at 80° for 90 min. A typical hydrolysis curve of the polysaccharide under these conditions is shown in Fig. 2. The amount of free *N*-acetylneuraminic acid never exceeded 33 % of the initial amount present regardless of the hydrolysis conditions employed. Stronger concentrations of acid resulted in excessive formation of humin. Weaker concentrations of acid gave a slower liberation of the *N*-acetylneuraminic and the longer time of heating required also resulted in considerable humin formation. The *C. freundii* polysaccharide is considerably more resistant to hydrolysis than is colominic acid (poly *N*-acetylneuraminic acid). Heating aqueous solutions of colominic acid to 100° readily yields *N*-acetylneuraminic acid, whereas heating the *C. freundii* polysaccharide under these conditions liberates negligible amounts of the acid. Moreover, mammalian mucopolysaccharides obtained from various sources readily release *N*-acetylneuraminic acid when heated in 0·1 N-sulphuric acid solution. Under these conditions little *N*-acetylneuraminic acid is liberated from the *C. freundii* polysaccharide. A more stable chemical linkage appears to exist between *N*-acetylneuraminic acid and the other constituents in the *C. freundii* polysaccharide than occurs in other known macromolecular materials which contain this substance.

Products of hydrolysis of Citrobacter freundii *polysaccharide.* Although *N*-acetylneuraminic acid can be detected in the *C. freundii* polysaccharide by colorimetric analytical procedures, it was necessary to establish its presence firmly by isolation and analysis. As a satisfactory procedure for hydrolysis of the poly-

saccharide which yields sufficient free *N*-acetylneuraminic acid to permit isolation had been devised, a study was undertaken to separate the acid from the products of hydrolysis by means of ion exchange chromatography (Barry, 1958).

A solution of 1·06 g. of purified *Citrobacter freundii* polysaccharide, containing 358 mg. *N*-acetylneuraminic acid, in 250 ml. 1 N-sulphuric acid was heated to 80° in a water bath. The amount of free *N*-acetylneuraminic acid was determined by the thiobarbituric acid method on samples of the hydrolysate taken at 15–30 min. intervals. After 120 min. the amount of free *N*-aeetylneuraminic acid had reached a maximum and then decreased slowly. Heating was stopped after 150 min. when 110 mg. of free *N*-acetylneuraminic acid was calculated to be present in the solution. The pH was adjusted to 6·8 by addition of saturated barium hydroxide and the barium sulphate precipitate was removed by centrifugation and washed with two

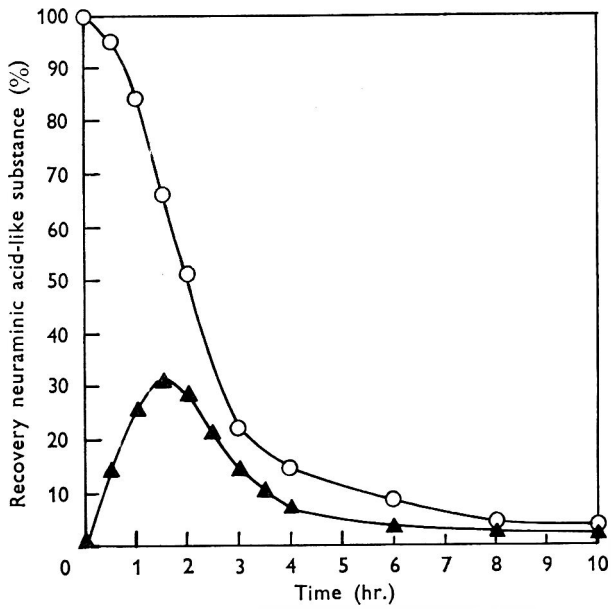

Fig. 2. Hydrolysis of *Citrobacter freundii* polysaccharide in 1·0 N-sulphuric acid at 80°. Ehrlich at 530 mμ, ○; thiobarbituric acid at 549 mμ, ▲.

separate 100 ml. portions of water warmed to 40° and centrifuged. The washings and original supernatant were combined and concentrated *in vacuo* at 40° to 200 ml. A quantitative recovery of the free *N*-acetylneuraminic acid was obtained. The solution was decolorized by addition of 200 mg. of charcoal (Darco-60), warmed to 40°, and filtered. The decolorization process was twice repeated using separate 100 mg. portions of charcoal. The filtrate was concentrated *in vacuo* at 40° to 50 ml. and the decolorization process repeated with 50 mg. of charcoal. Barium was removed by ion exchange from the final filtrate, which contained 102 mg. of *N*-acetylneuraminic acid, by passage through Amberlite resin IRC–50 (Rohm and Haas). The effluent and washings were combined and concentrated almost to dryness. The residue suspended in 10 ml. water was filtered through a fritted glass filter of medium porosity. The clear filtrate after lyophilization gave 587 mg. of an amber coloured powder containing 97 mg. of free *N*-acetylneuraminic acid.

To a 5 × 30 cm. column of Dowex 1-X8 resin (Biorad Lab.) in the formate form was placed 545 mg. of the purified hydrolysate, containing 90 mg. of free *N*-acetylneuraminic acid, dissolved in 5 ml. water. After the sample was adsorbed the column was washed with 600 ml. water and 10 ml. fractions were collected. The column was next eluted with dilute formic acid by the gradient elution technique. Analyses were made upon samples selected throughout the series for *N*-acetylneuraminic acid by the Ehrlich and Warren procedures. Absorbance measurements were made at 260 mμ to detect if substances which absorb in the ultraviolet emerged from the column.

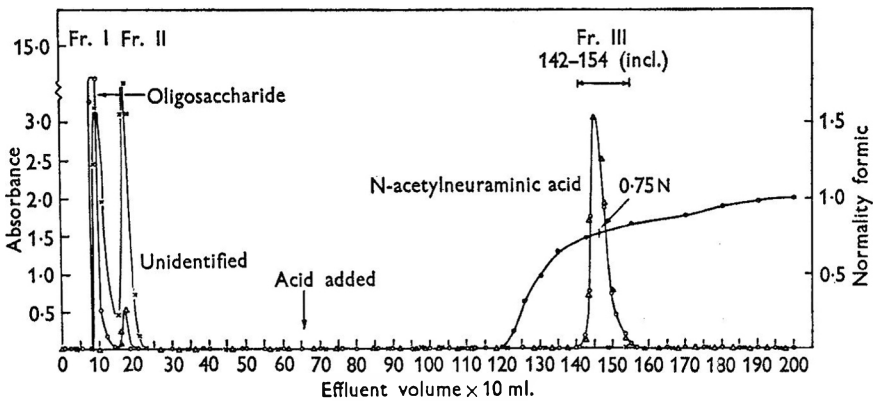

Fig. 3. Elution curve of 1 N-acid hydrolysate of *Citrobacter freundii* polysaccharide from Dowex 1-X8 resin column with formic acid. Formic acid concentration, ●; Ehrlich at 530 mμ, ○; thiobarbituric acid at 549 mμ, △; ultraviolet at 260 mμ, ×.

The analyses of the chromatographic separation are plotted in Fig. 3. Three distinct fractions emerged from the column. The first two fractions emerged at the beginning of the experiment. The curve of the third fraction to emerge was nearly symmetrical in shape and gave intense Ehrlich and Warren colour reactions. The curves obtained by these two analytical procedures were super-imposable by mathematical calculation (fitting second curve to a fixed point of first curve and multiplying each value by a factor) a fact which suggested that only a single substance was present.

The eluent fractions numbered 9 and 10 were combined and concentrated *in vacuo* to 10 ml. The solution was filtered and lyophilized. This fraction (Fr. 1) weighed 289 mg. and represented 53 % of the material originally placed on to the column. This substance is partially degraded polysaccharide and has an *N*-acetylneuraminic acid content of 22 % by the Ehrlich procedure. No colour is produced in the Warren test for free *N*-acetylneuraminic acid. 17 mg. (Fr. II) of the material was recovered from fractions 15–23 (inclusive). From fractions 142 to 154 (inclusive) 75 mg. (Fr. III) of the material was recovered (yield 13·8 %). It contained 90 % of *N*-acetylneuraminic acid (Warren procedure) and had 83·5 % of the free *N*-acetylneuraminic acid present in the original sample. Thus, 70 % of the material originally placed on the column was recovered in the three fractions.

Crystallization of Fraction III. Seventy mg. of Fr. III was dissolved in 0·3 ml. water and 4·0 ml. of 95 % ethanol added. Ethyl ether was added until a faint

turbidity developed (*c.* 4·0 ml.). After standing at 4° for 24 hr. an amorphous precipitate separated which was collected by centrifugation. The supernatant was decanted and petroleum ether added until the solution became turbid (1·5 ml.). The mixture was left at room temperature for a few hours, seeded with an authentic sample of *N*-acctylneuraminic acid and kept overnight at 4°. The heavy crystalline precipitate was collected by centrifugation and the supernatant decanted. Additional petroleum ether was added to the supernatant and a second crop of crystals obtained. This procedure was again repeated with the second supernatant. A total of 40·8 mg. was recovered. This substance, which crystallized in the form of needles, represented a yield of 57 %.

Properties of Fraction III. Crystalline Fr. III dissolved freely in water to give acidic solutions. When Fr. III was heated in dilute mineral acid humin was readily formed. A red colour with an absorption maximum at 530 mμ resulted upon heating the material with Ehrlich's reagent. When heated with Warren's reagent, a reddish colour was formed with an absorption maximum at 549 mμ.

Fraction III was dried to constant weight at 80° and the following elemental analysis obtained:

Substance analysed...		C	H	N	CH_3CO
Crystalline Fr. III	Found	42·52	6·42	4·39	13·48
N-Acetylneuraminic acid $C_{11}H_{19}NO_9$	Calculated	42·74	6·19	4·53	13·92

The optical rotation of the substance when measured at a concentration of 1·5 %, gave a value $[\alpha]_D^{23°} = -33·0° \pm 2·0°$. An $[\alpha]_D^{24°} = -31° \pm 2·0°$ value for *N*-acetylneuraminic acid has been reported by Blix, Lindberg, Odin & Werner (1956). Partition chromatography of 100 μg. of Fr. III on paper using three different solvent systems gave mobility values identical to those obtained with an authentic sample of *N*-acetylneuraminic acid. The chromatograms showed one spot and hence other derivatives of *N*-acetylneuraminic acid were absent. An infrared spectrum of 1·4 mg. of Fr. III taken in a 350 mg. pellet of potassium bromide is shown in Fig. 4B. It is evident that this spectrum is identical at every major point of reference to the one shown in Fig. 4C taken on an authentic sample of *N*-acetylneuraminic acid.

From the data it is concluded that the *Citrobacter freundii* polysaccharide is constituted in part of units of *N*-acetylneuraminic acid. Thus, the presence of a derivative of neuraminic acid in *C. freundii* O 5:H 30 initially determined by colorimetric tests (Barry, 1959) has been substantiated by isolation and characterization of the acid.

Properties of Fraction I and II. The amount of Fr. I which was obtained upon elution of the column varied with the time, temperature and acidity employed for the hydrolysis of *Citrobacter freundii* polysaccharide. Thus, the more drastic the conditions the smaller was the recovery of this fraction. No attempts have been made to characterize this oligosaccharide more fully. The amount of Fr. II recovered was always small under any conditions employed for hydrolysis. Although Fr. II gives tests for neuraminic acid its true chemical nature has not been investigated.

Analysis of 6 N-*acid hydrolysates of* Citrobacter freundii *polysaccharide.* Although the *C. freundii* polysaccharide had been thoroughly treated with trypsin during

purification there still remained 0·1–0·2 % of protein attached to the macromolecule as determined by the Folin-Ciocalteu test. It was of interest, therefore, to ascertain if the amino acids present were of the types commonly associated with bacterial products. A 6N-hydrochloric acid hydrolysate of 4 mg. of polysaccharide was prepared. After removal of the acid the residue was dissolved in a few ml. of water and passed through a 1 × 5 cm. charcoal (Darco-60) column. The column was washed with 20 ml. water. The eluents were combined and concentrated *in vacuo* to dryness and the residue examined for amino acids by two-dimensional partition paper chromatography. Twelve spots appeared on the chromatogram of the

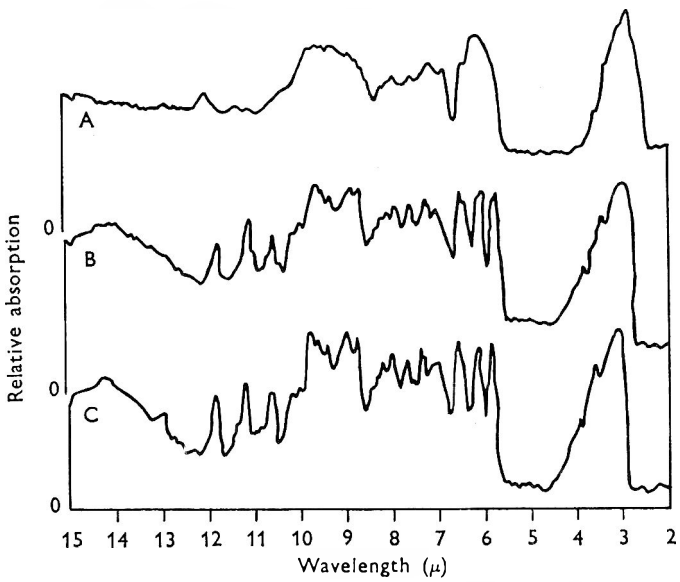

Fig. 4. Infrared spectrograms taken on 1·4 mg. of substance in 350 mg. of potassium bromide pellet; curve A, *Citrobacter freundii* polysaccharide; curve B, crystalline Fr. III; curve C, *N*-acetylneuraminic acid.

hydrolysate. Ten spots were identified as glucosamine, glycine, lysine, serine, threonine, alanine, valine, leucine and/or isoleucine and glutamic and aspartic acids. From the intensity of the colour produced by glucosamine and the two unknown spots it would appear that these materials are present in large amounts. One of the unidentified spots was yellow, whereas all the other spots on the paper were pink or grey.

Isolation of unknown compound which stains yellow with the ninhydrin reagent from hydrolysates of Citrobacter freundii *polysaccharide.* Proline and hydroxy-proline are the only amino acids which occur in hydrolysates of animal proteins that yield a yellow colour with the ninhydrin reagent. Certain cyclic imino or unsaturated amino acids isolated from plants which include derivatives of pipecolic acid (King, King & Warwich, 1950), proline (Hulme & Steward, 1955) cyclopropane (Burroughs, 1957), azetidine (Fowden, 1956) and γ-methylene glutamic acid (Done & Fowden, 1952) may give yellow complexes with the ninhydrin reagent. Moreover, 5-amino-imidazole-4-carboxamide obtained from *Escherichia coli* gives a yellow colour with this reagent (Greenberg & Spilman, 1956). However, the unknown compound pre-

sent in hydrolysates of the *C. freundii* polysaccharide differs from all these materials on the basis of its mobility in various systems. Thus, a study was undertaken to separate the unknown from the products of hydrolysis by means of ion exchange chromatography.

A solution containing 2·14 g. of purified *Citrobacter freundii* polysaccharide dissolved in 50 ml. of 6 N-hydrochloric acid was heated at 110° for 48 hr. in a sealed, 500 ml., round-bottomed flask. The mixture was filtered to remove humin. The filtrate was evaporated *in vacuo* to dryness to remove hydrochloric acid. The residue was suspended in 40 ml. of 95 % ethanol and insoluble material removed by centrifugation. The alcohol supernatant was concentrated *in vacuo* to dryness. The residue was redissolved in 5 ml. water and placed on to a 4·5 × 50 cm. Dowex AG 50W-X4

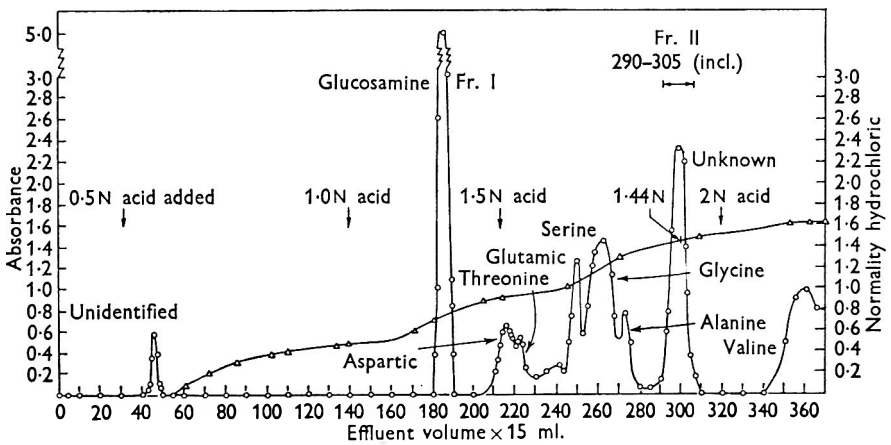

Fig. 5. Elution curve of 6 N acid hydrolysate of *Citrobacter freundii* polysaccharide from Dowex 50W-X4 resin column with hydrochloric acid. Hydrochloric acid △; ninhydrin at 570 mμ or at 440 mμ, ○.

(Biorad) column. After the sample was adsorbed, the column was washed with 500 ml. water and 15 ml. fractions were collected. The column was next eluted with dilute hydrochloric acid (0·5–2 N) by the gradient elution technique at the rate of 20 ml./hr. and 15 ml. fractions were collected. Analyses for amino acids were made on 1 ml. samples taken from every 5th fraction. The analyses of a typical chromatographic separation are plotted in Fig. 5. Several distinct peaks emerged from the column. Identification of the materials present in each peak was made by evaporation of a suitable sample to dryness *in vacuo*, redissolving it in a small amount of water and running one- or two-dimensional paper chromatograms.

The eluent fraction numbered 185 (Fr. I) contained a substance which on partition paper chromatographic analysis had a mobility identical to that of glucosamine. In addition, the material gave an intense reddish colour with an absorption maximum at 530 mμ when analysed for hexosamine by the procedure of Sorensen (1938).

The eluent fractions numbered 290–305 (inclusive) which gave a yellow colour in the ninhydrin analysis were combined and freed from hydrochloric acid by ion exchange, on Dowex 1-X8, concentrated *in vacuo* to a few ml. and 10 ml. of alcohol added. Ether was added dropwise to incipient turbidity and the solution placed overnight at 4°. The crystals which formed were collected by centrifugation and

dried in a desiccator. A recovery of 55 mg. was obtained. This amount represents an overall yield of 2·5 % (w/w) of the original weight of polysaccharide hydrolysed.

Properties of Fraction II. Crystalline Fr II. dissolves freely in water and in aqueous ethanol to give clear colourless solutions. Fr II. is stable to heating at 100° in 6 N or 12 N-hydrochloric acid. A yellow colour results upon heating the material with the ninhydrin reagent. The intensity of the absorbance at 440 mμ is 0·47 of that given by an equivalent weight of proline. Sulphur, phosphorus and acetyl are absent.

Fr. II was dried to constant weight at 100° and the following elemental analysis obtained:

Substance analysed...		C	H	N
Crystalline Fr. II	Found	49·61	7·69	9·55
$C_6H_{11}NO_3$	Calculated	49·64	7·64	9·65

From the data it can be seen that the empirical formula $C_6H_{11}NO_3$ represents closely the composition of the crystalline material. The material has a melting point of 143–144°. Primary amino nitrogen analysis by the nitrous acid method liberated 0·29 ml. of nitrogen gas (77 % of theory) from 30·6 μmole of the material. Although this value is low it is quite evident that a primary amino group is present in the molecule. Partition chromatography of 200 μg. of Fr. II on paper using several different solvent systems gave a single yellow spot after staining with the ninhydrin reagent. An R_F value of 0·44 was calculated in butan-1-ol + acetic acid + water (4 + 1 + 5, v/v) and 0·67 in phenol + water + 8-hydroxyquinoline (80 g + 20 g. + 0·4 g.). R_F values for proline were 0·35 and 0·77 in these systems.

Characterization of crystalline Fraction II. The structure of Fr II was determined from analysis of infrared absorption and nuclear magnetic resonance spectra and from a study of the products formed after oxidation and reduction.

Analysis of infrared absorption spectrum of Fraction II. An infrared absorption spectrum of crystalline Fr. II taken in a potassium bromide pellet is shown in Fig. 6. An interpretation of this spectrum made from data given by Bellamy (1962) is as follows:

The strong band at 5·82 μ is typical of that given by an undisturbed stretching vibration of a carbonyl situated between two methylene groups. The medium intensity band at 7·62 μ is typical of a carbonyl stretching absorption. The broad band between 6·0 and 6·4 μ is given by amino acids and the band at 6·28 μ can be assigned to an ionic carboxyl absorption. The weak but definite band at 4·78 μ is characteristic of the simple amino acids. Furthermore, the broad band in the vicinity of 3·2 μ is typical of NH_2 stretching frequencies and NH stretching vibrations. Thus, the three oxygen atoms present in Fr. II appear to be bound to carbon atoms as C=O and COOH groups.

Oxidation of Fraction II. To 0·1 ml. of 10 % sulphuric acid solution containing 2 mg. of crystalline Fr. II was added 0·1 ml. of 1 % potassium permanganate solution in 10 % sulphuric acid. The purple colour was discharged upon warming the mixture to 60°. Further addition of oxidizing reagent was made at 60° until the purple colour persisted. In all 1·5 ml. of the reagent was added. Sulphate ion was removed with solid barium carbonate and the supernatant concentrated to dryness *in vacuo*. The residue was dissolved in 2 ml. of water and 1 ml. reconcentrated to dryness. The residue was dissolved in 0·1 ml. of water and analysed by two-dimensional partition paper chromatography. A pink and a yellow spot appeared

after staining with the ninhydrin reagent. The pink spot had a mobility identical to that given by aspartic acid. The yellow spot was unchanged material. It is concluded that Fr. II has a structure consisting of at least four carbon atoms arranged in a chain and that the carbonyl and carboxyl groups are separated from each other by two carbon atoms.

Reduction of Fraction II. Although aspartic acid was obtained upon oxidation of Fr. II no information was provided concerning the location of the amino group in the intact molecule. The position of the latter was ascertained from an analysis of the products obtained after hydrogenation of Fr. II.

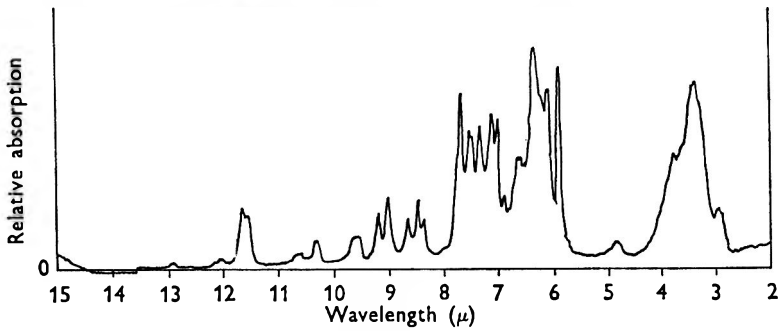

Fig. 6. Infrared spectrogram taken on 1·5 mg. of Fr. II in 800 mg. of potassium bromide pellet.

A solution containing 10·9 mg. of Fr. II was hydrogenated. After completion of the hydrogenation the suspension was filtered to remove the catalyst and the filtrate concentrated *in vacuo* at 50° to dryness. The residue was dissolved in 0·5 ml. of 50 % ethanol, ether added until incipient turbidity (*c.* 4 ml.) and the mixture placed at 4°. The crystals which formed were collected by centrifugation and dried in a desiccator. A yield of 7·5 mg. of material was obtained.

The reduced compound had a melting point of 201° and contained 9·41 % of nitrogen. $C_6H_{13}NO_3$ has a calculated nitrogen content of 9·52 %. If the carbonyl bond of Fr. II was reduced to a hydroxyl group 1·88 ml. of hydrogen gas would be absorbed under the experimental conditions employed. As 1·75 was absorbed it is concluded that only this bond was reduced.

Examination of the infrared absorption spectrum of reduced Fr. II taken in a potassium bromide pellet revealed the absence of carbonyl absorption bands at 5·82 and 7·62 μ present in the unreduced compound. In addition, a strong band was present at 2·70 μ which is typical for the OH valence stretching vibrations of an unbonded hydroxyl group.

Two-dimensional partition paper chromatographic analysis of 350 μg. of reduced Fr. II gave one strong grey and one faint blue spot after staining with the ninhydrin reagent. As no yellow spot appeared it is apparent that the unknown was completely reduced. The blue spot had a mobility and colour identical to that given by norleucine. Thus, it is indicated that the unknown Fr. II has a structure consisting of six carbons arranged in a chain. The grey spot had a colour and mobility which differed from any known amino acid.

Periodate oxidation of the reduced Fr. II. Additional evidence to establish the position of the amino group in Fr. II was obtained by exposure of the reduced com-

pound to periodate. If the hydroxyl and amino groups are on adjacent carbons this oxidizing reagent will cleave the two carbons and also result in the release of ammonia. Whereas, if the two groups are separated from each other, by one or more carbons, no reaction will occur.

To separate 0·2 ml. portions of periodate reagent (0·1 M-sodium periodate dissolved in 0·1 M-acetate buffer of pH 5·0 or in 0·1 phosphate buffer of 7·0) was added 0·1 ml. (500 μg.) of the reduced compound dissolved in water. After standing for

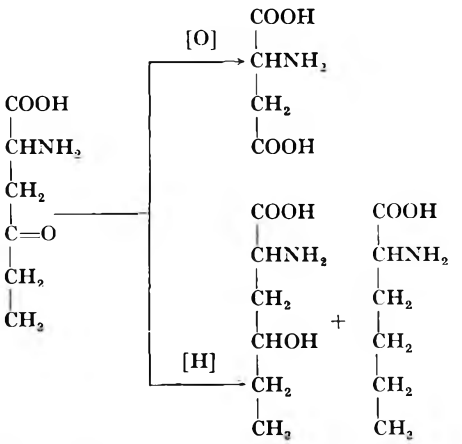

Fig. 7. Products formed after oxidation and reduction of Fr. II.

5 min. at 26° a portion of the mixture containing 50 μg. of the material was removed and analysed by partition paper chromatography. Control tubes containing 3-hydroxy-norleucine and 6-hydroxy-norleucine were also included. After staining with the ninhydrin reagent spots appeared for the reduced Fr. II and 6-hydroxy-norleucine. However, the 3-hydroxynorleucine spot did not appear. It is obvious, therefore, that the NH_2 and OH groups in reduced Fr. II are not on adjacent carbon atoms. Furthermore, the amino group must lie on the carbon α to the carboxyl group.

The results of the chemical studies clearly reveal that the unknown Fr. II is 4-oxo-norleucine and that the principal product obtained upon reduction is 4-hydroxy-norleucine. A summary of the chemical products formed in the oxidation and reduction experiments of Fr. II is outlined in Fig. 7.

Analysis of nuclear magnetic resonance spectrum of Fraction II. The position of the protons on the carbon atoms of Fr. II was ascertained from analysis of nuclear magnetic resonance spectral data. A spectrum of the compound taken on 10 mg. of material in deuterium oxide is shown in Fig. 8. Two sets of interacting protons are clearly separated from one another. One set shown by the triplet at 1·07 p.p.m. and the quartet at 2·65 p.p.m. give a typical ethyl ($—C_2H_5$) pattern. The other shown by the doublet at 3·27 p.p.m. indicates a methylene ($—CH_2—$) group adjacent to a carbon with a single hydrogen atom. The triplet at 4·03 p.p.m. represents absorbance by a single hydrogen atom ($—\overset{|}{C}H$) flanked by carbon atoms which have a total of two hydrogens. The singlet at 4.30 p.p.m. represents absorbance due to water, resulting from the exchange of deuterium atoms with hydrogen

in the amino and hydroxyl of the carboxyl group. The line intensities and the integral confirm the groupings $-C_2H_5$, $-CH_2$ and $-\overset{|}{\underset{|}{C}}H$. The insolubility of Fr. II in other solvents employed in nuclear magnetic resonance analysis did not permit resolution of the hydrogen on the oxygen and nitrogen atoms.

It is concluded therefore, both from the results of the chemical studies and from the analysis of the spectral data that the structure of Fr. II conforms to 4-oxo-norleucine.

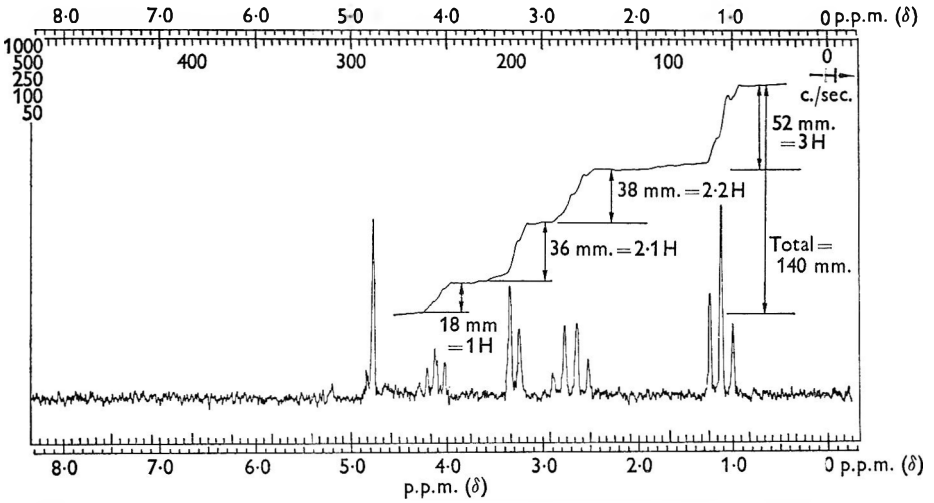

Fig. 8. Nuclear magnetic resonance spectrogram taken on 10 mg. of crystalline Fr. II in deuterium oxide at a sweep width of 500 c./sec. at 60 Mc and sweep time of 500 sec. Spectrum amplification 25 and integral amplification 80. Gain cut by 1/10 at 4·63 p.p.m.

Isolation of a polysaccharide from Salmonella dahlem. A polysaccharide constituted of 32 % of *N*-acetylneuraminic acid and 28 % of glucosamine was isolated from *S. dahlem* bacteria by the use of a procedure identical to that employed in the isolation of the *Citrobacter freundii* polysaccharide. Moreover, hydrolysates of the material gave a compound which stains yellow with the ninhydrin reagent as determined by paper partition chromatographic analysis. The mobility of this material was identical to that of the 4-oxo-norleucine isolated from hydrolysates of the *C. freundii* polysaccharide. It was concluded, therefore, that both *S. dahlem* and *C. freundii* O 5:H 30 contain a similarly constituted polysaccharide.

Analysis of 6 N-*hydrolysates of amino sugars and of other polysaccharides.* It was of interest to determine if 4-oxo-norleucine is a product which results from rearrangement of the *N*-acetylneuraminic acid or of the glucosamine during hydrolysis of the *Citrobacter freundii* polysaccharide. In addition, a study was made of hydrolysates of various polysaccharides containing derivatives of neuraminic acid and of the Vi antigen which is produced by *C. freundii* O 5:H 30. Paper partition chromatography of hydrolysates of *N*-acetylneuraminic acid, glucosamine, colominic acid, group C *Neisseria meningitidis* hapten and the Vi antigen did not give a spot with a colour and mobility corresponding with that of 4-oxo-norleucine found in hydrolysates of the *C. freundii* polysaccharide.

DISCUSSION

Of the few polysaccharides containing derivatives of neuraminic acid which have been found in bacteria, the best known is colominic acid (Barry, 1958) a substance constituted solely of units of *N*-acetylneuraminic acid. This homopolysaccharide is elaborated into the culture media by strains of *Escherichia coli* which have a K1 serotype (Barry, 1959; Barry *et al.* 1960). From the results presented in this communication it appears that the enteric micro-organism *Citrobacter freundii* O5:H30 produces considerable amounts of a nitrogenous heteropolysaccharide rich in neuraminic acid. This is a new and hitherto undescribed polysaccharide constituted primarily of a unit of *N*-acetylneuraminic acid, two of glucosamine and one of an uncharacterized acid labile nitrogenous constituent. Thus, this serologically active polysaccharide differs markedly in chemical composition from the serologically inactive colominic acid. In addition, it appears that a polysaccharide which is constituted similarly to the *C. freundii* polysaccharide is produced by *Salmonella dahlem.* It is suggestive that the closely related organism, *S. djkarta*, which contains neuraminic acid, also produces a similar polysaccharide. Thus, the contention that colominic acid is produced by *C. freundii* (*E. coli* 5396/38) and related strains *S. djkarta* and *S. dahlem* (Westphal, Kauffmann, Luderitz & Stierlin, 1960; Kauffmann, Luderitz, Stierlin & Westphal, 1960) is not substantiated by the present studies. Moreover, the serological relationship previously shown between *C. freundii* O5:H30, *S. dahlem* and *S. djkarta* (Barry *et al.* 1962) now suggests that a chemical relationship also exists between these micro-organisms.

Citrobacter freundii O5:H30 (*E. coli* 5396/38), *Salmonella typhosa* Ty2 and Ballerup 7851/39 have for many years been known to be producers of Vi antigen, a polymer of *N*-acetamidogalacturonic acid (Heyns *et al.* 1959). Although *N*-acetylneuraminic acid forms a considerable portion of the chemical composition of *C. freundii* O5:H30, its detection in *S. typhosa* Ty2 and Ballerup 7851/39 was unsuccessful (Barry *et al.* 1962). Thus, the occurrence of a similarly constituted polysaccharide containing neuraminic acid in *C. freundii* O5:H30 and in the non-Vi *S. dahlem* and its absence in *S. typhosa* Ty2 and Ballerup 7851/39 indicates that this polysaccharide is unrelated to Vi antigen. Moreover, the chemical constitution and properties of the *C. freundii* polysaccharide containing *N*-acetylneuraminic acid differ markedly from those of the Vi antigen.

The isolation of 4-oxo-norleucine from hydrolysates of *Citrobacter freundii* polysaccharide and its detection in hydrolysates of a polysaccharide obtained from *Salmonella dahlem* indicates that this new and hitherto undescribed amino acid can be obtained from different bacteria. However, the distribution of this new component among bacterial species remains largely unknown. At this time it is not possible to conclude whether 4-oxo-norleucine or a derivative of this material forms part of the intimate structure of the *C. freundii* polysaccharide or results as a degradation product of the uncharacterized acid labile third component. In view of the well-known degradation of aldohexoses to levulinic acid upon heating in mineral acids, it might appear that the origin of the 4-oxo-norleucine is an amino sugar. Neither *N*-acetylneuraminic acid nor glucosamine rearrange to 4-oxo-norleucine upon heating in acid. In addition, poly *N*-acetamidogalacturonic acid (Vi antigen), poly *N*-acetylneuraminic acid (colominic acid) and a polysaccharide

largely composed of neuraminic acid (group C *Neisseria meningitidis* hapten) fail to yield 4-oxo-norleucine upon heating in acid.

The chemical investigations of the polysaccharide obtained from *Citrobacter freundii* and its hydrolytic products have revealed that derivatives of neuraminic acid are associated with other nitrogenous monosaccharides in polysaccharides of bacterial origin. Moreover, such mucopolysaccharides in association with amino acids can constitute part of the basic structural elements of various bacteria.

The synthesis of 4-oxo-norleucine was recently accomplished (Barry & Roark, 1964; to be published). The synthetic material is identical to the isolated bacterial product.

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A Method for the Purification of Bacterial Flagella by Ion Exchange Chromatography

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SUMMARY

A method is described for purification of bacterial flagella by elution of these organelles from a DEAE cellulose column by NaCl gradients.

INTRODUCTION

Preliminary to a study of the structure and chemistry of bacterial flagella it was essential to devise a simple and reproducible method for the purification of this organelle. Repeated attempts at purification by using the published methods of differential centrifugation (Weibull, 1950; Kobayashi, Rinker & Koffler, 1959) did not yield preparations which would satisfy rigorous criteria of purity with any degree of reproducibility. Diethylaminoethyl cellulose (DEAE cellulose) has been successfully used for purification of viruses (Wilson, 1962), and this observation tempted us to use the property of ionic charge for the purification of flagella. A new method of purification of bacterial flagella has been developed based on the observation that flagella will adsorb on to DEAE cellulose columns and may be differentially eluted by salt solutions. Attempts were also made to use the property of antigenic specificity for purification of the isolated structures. This approach was abandoned since complete dissociation of the antigen-antibody complex was not easily achieved.

METHODS

Organisms and growth conditions. *Spirillum serpens* was used in most of the experiments. *Proteus vulgaris* and *Bacillus subtilis* SB19 were also investigated. The organisms were grown in 40 l. carboys in complex media (nutrient broth or N.Z. case, an enzymic digest of casein; Sheffield Farms Co., Norwich, New York) with mild aeration. The cultures in late log phase were harvested at 2°.

Isolation of flagella. The organisms were resuspended in cold 0·01 M-phosphate buffer (pH 7·0) and deflagellated mechanically in the Lourdes Multi-Mix Homogenizer (16,000 rev./min. for 60 sec.) at 2°. The cells were removed by centrifugation at 5000 *g* for 30 min. The cell pellet was washed once with one half the initial volume of buffer, and the cells again sedimented by centrifugation. The pooled supernatant fluids were subjected to centrifugation at 105,000 *g* for 30 min. to sediment the flagella. The sedimented flagella were resuspended in 0·5 ml. buffer by allowing them to stand in the refrigerator overnight. Initial trials with water as the suspending medium for isolated flagellar suspensions of *Spirillum serpens* resulted in significant dissociation of the structures; phosphate buffer (0·01 M; pH 7·0) proved to be a suitable suspending medium.

Analytical methods. All the chemical determinations used were modifications of existing procedures. The volumes prescribed in published methods were scaled down, thus increasing the sensitivity of the analyses while simultaneously decreasing the volume of sample required. In the orcinol method (Schneider, 1955) dichromatic readings were made. All analyses were read in a Zeiss spectrophotometer. The methods used were the Lowry (Lowry, Roseborough, Farr & Randall, 1951) for protein; Fiske & SubbaRow for phosphorous (Fisk & SubbaRow, 1925); orcinol for ribonucleic acid (RNA) (Schneider, 1955) and the diphenylamine for deoxyribonucleic acid (DNA) (Schneider, 1955).

Serological methods. Anti-flagellar sera were prepared by intravenous injection of intact flagellated organisms into rabbits. The somatic antibodies were adsorbed from the antisera with deflagellated cell suspensions which had been heated at 100° for 60 min. and washed three times with buffer. Adsorption was carried out at least twice after the sera were negative for somatic antibodies.

Antisera were also prepared against purified flagella. The purified preparations (1·5 mg. protein/ml.) were injected with Freund's incomplete adjuvant into the foot pads of rabbits. Antisera prepared with highly purified flagella showed a low degree of cross-reactivity with homologous somatic antigen, indicating common antigenic sites between flagellar and somatic constituents. The somatic antibodies were removed by adsorption (see above) without a diminution of anti-flagellar titre. Agglutination inhibition (Kabat & Mayer, 1961) was used for the detection of flagellar antigen by using intact flagellated organisms in formalinized saline buffer as the agglutination indicator system. By this procedure less than 1 μg. flagellar protein/ml. could readily be detected.

Preparation of DEAE cellulose columns. The DEAE cellulose (Schleicher and Schüll Co.) was washed with several volumes of N-NaOH and then with water. Chromatography columns were packed with this DEAE cellulose slurried in 2 M-NaCl under atmospheric pressure. Preparation of the columns in high salt solutions resulted in even packing without any channelling. The columns were washed until chloride-free and were then saturated with 0·01 M-phosphate buffer (pH 7·0). The chromatography was carried out at room temperature. Crude flagellar suspensions were adsorbed on the DEAE cellulose and the columns were developed with increasing NaCl concentrations in buffer.

Electron microscopy. Cells and flagellar preparations were examined after negative staining with phosphotungstic acid (Kerridge, Horne & Glauert, 1962); a solution (1 % w/v) of uranyl acetate was also used.

RESULTS

Preliminary experiments showed that crude flagellar suspensions from *Spirillum serpens* were adsorbed by DEAE cellulose (pH 7·0) in phosphate buffer and were not eluted by 20 column volumes of this buffer. Elution of the flagella was achieved by increasing the molarity of NaCl in the buffer eluant. Figure 1 is a typical elution diagram of flagella from DEAE cellulose. In this experiment 15 ml. of a crude suspension of flagella (the pellet sedimented at 105,000*g* in 30 min.) in buffer containing 3·02 mg. protein/ml. were passed through a DEAE cellulose column (1·8 × 12 cm.) previously saturated with buffer. The column was washed with 100 ml. buffer and stepwise increases in NaCl in the buffer were used as developing agent.

Elution was carried out at 1·5 ml./min.; 10 ml. fractions were collected. Absorbancy of the eluates was measured in a Zeiss spectrophotometer at 280 mμ. The optical density readings are not directly proportional to light absorption since the light scattered is very significant in the peak fractions because of the Tyndall effect of the flagellar suspensions. Protein and RNA determinations were made on all fractions. The orcinol determinations were negative with the exception of fractions 41 to 44, thus explaining the high absorbancy at 280 mμ with essentially negligible protein in these tubes. Further analyses of this 0·8 M-NaCl peak showed it to consist of RNA. Total protein recovered in the peak fractions (39·7 mg.) accounted for 88%

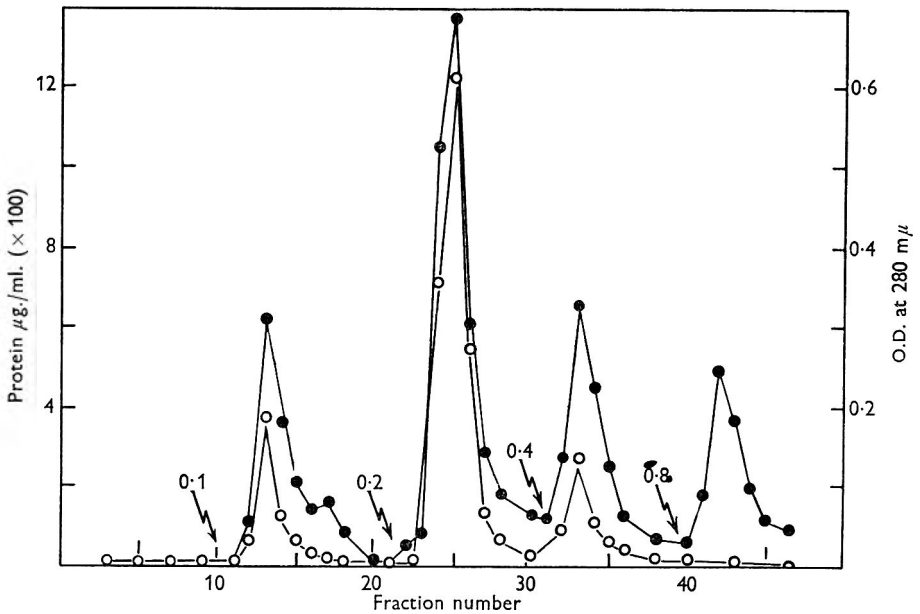

Fig. 1. Elution pattern of *Spirillum serpens* flagella from a DEAE cellulose column. The column was developed by stepwise increases of NaCl concentration (0·1 M, 0·2 M, 0·4 M, 0·8 M) in 0·01 M-phosphate buffer (pH 7·0). ○, Protein; ●, optical density.

of the protein passed through the column. Electron microscopy of the samples from peak fractions showed flagellar fragments in all the three major peaks—0·1 M, 0·2 M and 0·4 M-NaCl. The 0·8 M peak showed no structures recognizable as flagella.

Three possible explanations for the presence of the discrete peaks noted in Fig. 1 were considered: (1) three different species of flagella are made by the organisms; (2) there is a discontinuous size distribution of flagella produced by the organisms or by the deflagellation method; (3) there is a continuous size distribution of flagella produced either by the organisms or by the deflagellation method.

The first hypothesis was tested serologically by using antiflagellar antibodies prepared against the purified flagella eluted with 0·2 M-NaCl. It was found that the equivalence point for antigen-antibody reaction was identical for all three peaks. The equivalence point was determined initially for the 0·2 M-NaCl flagellar fractions with homologous antibody. The same equivalence point was obtained with the 0·1 and 0·4 M-NaCl eluates against the same serum (0·2 M-NaCl fraction antiserum).

The results of this experiment argue against the hypothesis of three species of flagella produced by *Spirillum serpens*.

Hypotheses (2) and (3) were tested by eluting the flagella from the DEAE cellulose column with a continuously increasing NaCl gradient. If hypothesis (2) were correct, one should obtain three discrete peaks even if elution were carried out by a gradient, whereas a continuous size distribution would result in a single peak. Figure 2 illustrates an experiment in which a crude *Spirillum serpens* flagellar suspension was absorbed on to a 1·8 × 19 cm. DEAE cellulose column, and elution carried out by using a concave gradient of NaCl (500 ml. of salt-free buffer in the

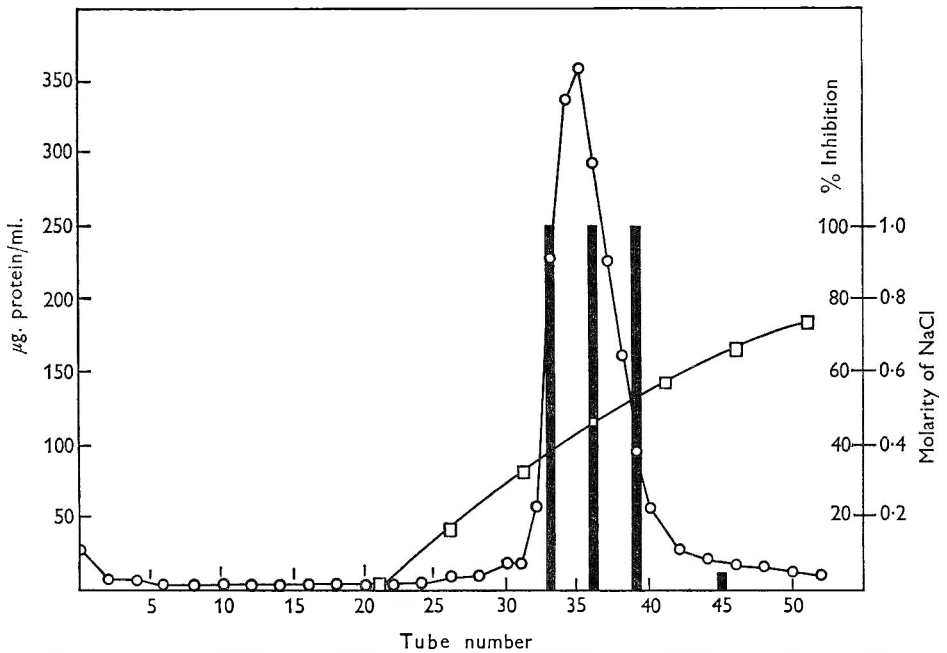

Fig. 2. Elution pattern of *Spirillum serpens* flagella from a DEAE cellulose column. The column was developed with a NaCl gradient in buffer. □, NaCl concentration; ○, protein. The solid bars denote the % inhibition of agglutination (see text).

mixing vessel and 500 ml. of 2 M-NaCl in buffer in the reservoir). Elution was carried out at 1·5 ml./min. and 10 ml. fractions were collected. The gradient was started after 200 ml. of buffer had been used to wash the column. It is evident from examination of the figure that a single peak was obtained, thus indicating that hypothesis (2) was incorrect. The data suggest, therefore, that during the deflagellation procedure a continuous distribution of flagellar sizes was produced.

The antigenic homogeneity of the flagella eluted by the gradient method was also tested by the agglutination-inhibition technique. The results of such an analysis are plotted in Fig. 2 as % inhibition of agglutination of the indicator system by the eluate fractions. It is evident that inhibition of agglutination occurred only in the peak fractions; that is where flagella were observed in the electron microscope.

These peak fractions (Fig. 2) were pooled and the flagella harvested by centrifugation. A sample of the resuspended flagella preparation was chromatographed again on a DEAE cellulose column (1·8 × 14 cm.) with the conditions of gradient

elution as in Fig. 2. Figure 3 illustrates the results obtained. It is evident that rechromatography resulted in a reproducible elution of the flagella by the same salt concentration as in the original column passage.

After elution from the column, the flagella may be harvested by centrifugation at 105,000*g* for 30 min. Analyses for protein in the supernatant fluid and pellet revealed trace amounts (usually no more than 2 % of total) of protein remaining in the supernatant fraction. These supernatant fractions, however, were still capable of agglutination inhibition, implying that the residual non-sedimented protein was of flagellar origin. It was sometimes necessary to de-salt the sedimented flagellar suspensions. This was readily achieved by passage of the flagellar suspension through a Sephadex (Pharmacia Fine Chemicals Inc., Uppsala, Sweden) column.

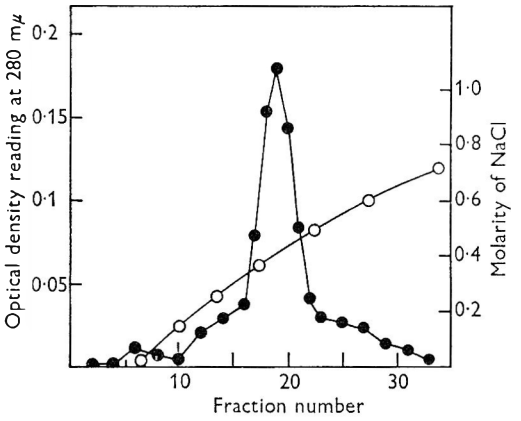

Fig. 3

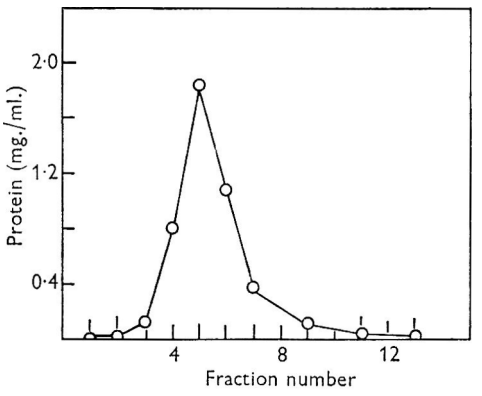

Fig. 4

Fig. 3. Elution pattern of a sample of the flagella of *Spirillum serpens* obtained in the experiment recorded in Fig. 2 from DEAE cellulose. ○, NaCl concentration; ●, optical density.

Fig. 4. Elution pattern of *Spirillum serpens* flagella from Sephadex G 50 column. Column was developed with 0·005 M-NaCl.

Figure 4 illustrates an elution diagram of such an experiment. Four ml. of a suspension of flagella recovered from DEAE cellulose eluates containing 2·65 mg. protein/ml. were passed through a Sephadex G 50 (medium) column (1·8 × 20 cm.) saturated with 0·005 M-NaCl. The column was developed with 0·005 M-NaCl; 2 ml. fractions were collected and protein determinations were made on all fractions. The flagella were eluted as a single uniform peak. In this experiment 9·65 mg. protein were recovered in fractions 3–9, indicating a 91 % recovery of the total protein placed on the column. When structural integrity of the flagella is not required, water may be used as eluting agent in the Sephadex treatment. Alternatively, volatile salts such as ammonium acetate or triethylammonium acetate may be used as developer.

The DEAE cellulose NaCl-gradient elution procedure described above for *Spirillum serpens* has been tested with flagella suspensions isolated from *Bacillus subtilis* and *Proteus vulgaris*. The elution patterns obtained with the flagella from these organisms were essentially identical with those obtained for *S. serpens*, suggesting that the method may be used for the purification of flagella from any bacterial strain.

The evidence presented in this paper for the purity of the preparations obtained by the method described is the uniformity of elution pattern, from both DEAE cellulose and Sephadex, the antigenic homogeneity of the preparations, and the cleanness of the electron microscope pictures. In addition, the column peak fractions were free from nucleic acids and phosphorous (determined after elution from Sephadex with water) in contrast to the crude flagellar suspension. These data in themselves are insufficient to establish the degree of purity of the flagellar preparations; for this, extensive chemical and physical data are required. This information will appear in a subsequent communication.

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Analysis by Transduction of Mutations affecting Penicillinase Formation in *Staphylococcus aureus*

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SUMMARY

Seventy-five mutants with alterations in penicillinase formation were isolated from a strain of *Staphylococcus aureus* inducible for penicillinase. The mutants fell into three main categories on the basis of penicillinase activity and inducibility: (i) microinducible mutants which formed decreased amounts of penicillinase but retained the property of inducibility; (ii) penicillinase-negative variants which produced no detectable penicillinase and which showed no effect of inoculum size on penicillin resistance; (iii) strains with a wide range of penicillinase activities that produced the enzyme constitutively. Treatment of the wild-type strain with ethylmethane sulphonate increased the frequency of occurrence of microinducible and constitutive mutants but did not alter the incidence of the penicillinase-negative variants which were present in all cultures at a frequency of about 10^{-3}. Representative mutants of each class were examined for ability to revert to wild type and to give wild-type recombinants in transductional crosses. The constitutive strains and the microinducible strains behaved like point mutants in that they reverted and in that they gave wild-type recombinants. The penicillinase-negative mutants, however, behaved differently in that they were not observed to revert nor did they give wild-type recombinants in crosses, either with one another or with microinducible or constitutive mutants. A naturally occurring penicillinase-negative strain of *S. aureus* behaved similarly to the penicillinase-negative mutants in these respects. The possibility that the penicillinase region in *S. aureus* is associated with a plasmid and thus inherited extrachromosomally is considered and discussed. The properties of the penicillinase-negative variants could be explained as resulting from the loss of such a plasmid. Consistent with the plasmid hypothesis is the finding that ultraviolet irradiation of transducing phage produced an exponential decline of transducing titre for penicillinase; against it is the failure of acridine orange to increase the frequency of the penicillinase-negative variants.

INTRODUCTION

Naturally occurring penicillin-resistant staphylococci owe their resistance to the production of penicillinase, and the proportion of strains which produce this enzyme has risen steadily since the introduction of penicillin. However, naturally occurring penicillinase-negative staphylococci have never been observed to mutate to penicillinase production (Barber, 1957). Thus, it is currently accepted that the increase in the prevalence of penicillinase-producing strains is primarily the result of selection by penicillin, though phage-mediated transduction of penicillinase-producing capacity

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may be a contributory factor (Ritz & Baldwin, 1961). On the other hand, it has been observed that penicillinase-positive strains give rise frequently to penicillinase-negative variants (Barber, 1949; Bondi, Kornblum & de Saint Phalle, 1953; Fairbrother, Parker & Eaton, 1954). These strains are usually stable in that, like wild-type penicillinase-negatives, they do not revert to penicillinase production (Fairbrother, 1956). The present work was undertaken with the primary purpose of elucidating the genetic difference between penicillinase-positive and -negative strains of *Staphylococcus aureus* and the mechanism of 'loss' of penicillinase-forming ability. Since staphylococcal penicillinase is an inducible enzyme (Geronimus & Cohen, 1957), it was hoped also to study genetically the mechanism of its induction.

The experimental approach to the problem was based on the following argument and was greatly aided by the availability of bacteriophages active in the transduction of the capacity to produce penicillinase to negative strains (Ritz & Baldwin, 1961; Pattee & Baldwin, 1961). Apparently the genetic information required for penicillinase synthesis is not present in the penicillinase-negative strains to such a degree of completeness that a single-step mutation can fully restore penicillinase-forming ability. It may be asked, however, whether any of the necessary information is present in these strains and if so, to what extent. A potentially similar situation was studied by Luria, Adams & Ting (1960), who found that a lac− strain of Shigella which did not mutate to lac+ nevertheless had a considerable proportion of the genetic information required for lactose fermentation because lac+ recombinants resulted when it was used as a donor in transductional crosses with various *Escherichia coli* lac− point mutants. Further, this strain of Shigella carried intact the locus controlling the inducibility of the enzymes of lactose fermentation. Similarly, penicillinase-negative strains of *Staphyloccus aureus* should be able, if they carry any of the requisite genetic information, to repair by recombination a proportion of point-mutational penicillinase losses. On the other hand, if penicillinase-negative strains possess none of the information, no penicillinase-positive recombinants would be expected from crosses with point mutants.

Much of the work to be described has involved the development of media and methods and the isolation and characterization of mutants of *Staphylococcus aureus* in which penicillinase formation was affected. Transductional crosses have been made with several pairs of the mutants isolated; however, the recombination data thus obtained must be regarded as preliminary. As yet, no penicillinase-positive recombinants have been observed in crosses where one or both parents were naturally occurring penicillinase-negative. The formation of penicillinase-positive recombinants has, however, occurred in crosses between pairs of strains with point mutations in the penicillinase locus.

METHODS

Media. Preparation and composition of CY media based on CCY medium (Gladstone & Fildes, 1940) are described in Table 1. The designations, 0·3 CY, 0·5 CY and CY refer to media containing 0·3, 0·5 and 1·0 % (w/v), respectively, of hydrolysed casein and of yeast extract. The 0·3 CY medium gave rather poor growth with the staphylococcal strains used but, with its calcium chloride supplement, permitted excellent growth and plaque formation of the phages. The 0·5 CY medium gave good growth with all of the strains used, and C Y medium, unsupple-

mented with calcium, was inhibitory for most of the phages and was used to prevent the destruction of transductants in crosses mediated by non-lysogenizing phage. It was also used when especially dense cultures were desired, e.g. in penicillinase induction experiments.

Andrade indicator agar (Kogut, Pollock & Tridgell, 1956) was supplemented with yeast extract, 0·5 % (w/v), starch, 0·3 % (w/v), and, usually, benzylpenicillin, 0·10 μg./ml.

Table 1. *Preparation and composition of CY media and buffer*

Media were prepared with de-ionized water and autoclaved at 115° for 25 min. in screw-capped bottles for storage.

	Phage buffer; for harvesting, dilutions, and storing of phage	0·3 CY; for propagation, titration, and spot testing of phage	0·5 CY; for routine culture medium	CY; for selection of transductants by using non-lysogenizing phages and for penicillinase induction
β-Glycerophosphate*	0·06 M	0·06 M	0·06 M	0·12 M
$MgSO_4$	0·001 M	0·001 M	0·001 M	0·001 M
$CaCl_2$†	0·004 M	0·004 M	—	—
NaCl	0·085 M	—	—	—
Glucose†	—	—	0·028 M	0·028 M
Gelatin	0·1 % (w/v)	—	—	—
Trace metals‡	—	0·02 ml./l.	0·02 ml./l.	0·02 ml./l.
Yeast extract (Difco)	—	0·3 % (w/v)	0·5 % (w/v)	1·0 % (w/v)
Acid hydrolysed casein (Difco)	—	0·3 % (w/v)	0·5 % (w/v)	1·0 % (w/v)
Agar (Difco), when required	—	1·5 % (w/v)	1·5 % (w/v)	1·5 % (w/v)

* Added after autoclaving when agar was present. † Added after autoclaving. ‡ 0·5 % (w/v) $CuSO_4$; 0·5 % (w/v) $ZnSO_4$; 0·5 % (w/v) $FeSO_4$; 0·2 % (w/v) $MnCl_2$; 10 % (v/v) HCl.

Phage buffer. The composition of phage buffer is shown in Table 1. It was used for harvesting, storing, and diluting phage lysates.

Acid-base indicator agar. Agar, 1·5 % (w/v), with benzylpenicillin, 10 mM and an acid-base indicator, N-phenyl-1-naphthylamine-azo-0-carboxybenzene (mM) was poured in 200 ml. quantities on plate glass squares, using a 32 × 32 cm. Perspex frame. This particular indicator was chosen because its acid form is insoluble in water; acid production was thus indicated by sharply localized non-diffusing haloes.

Starch iodide indicator agar. Agar, 1·5 % (w/v), with benzylpenicillin 0·5 mM, iodine 1·0 mM, and starch (Analar) 0·3 % (w/v), was poured in 120 ml. quantities on the plate glass squares.

Organisms

Bacterial strains. Staphylococcus aureus was used exclusively in this work. Strain 524 SC (Rogers, 1953) is a laboratory strain; strain U-9 was kindly provided by Dr P. A. Pattee; strains NCTC 8510, 8511, and 8325 are the propagating strains for phages 7, 53, and 47, respectively, of the International Typing Series. The series of propagating strains was obtained from the Central Public Health Laboratories, Colindale, London, N.W. 9. All strains were stored on blood agar slopes and were

renewed from dried preparations (Stamp, 1947) at 6-month intervals. See Table 2 for relevant phenotypic characteristics.

Parental substrains. See Table 2. A heterogenetic penicillinase-inducible substrain of 8325, designated 8325–18, was constructed by transduction with phage 53α using strain 524 sc as the donor. This was done to make possible the isolation of a group of secondary penicillinase-negatives derived from the original wild type, 8325. A streptomycin-resistant mutant (0·5 mg./ml.) designated s-1 was selected after ethylmethane sulphonate (EMS) treatment of 8325–18. From these two strains were isolated all of the mutants used in this work. Strain Y-4 is a yellow

Table 2. *Characteristics of wild-type strains and of parental substrains of* Staphylococcus aureus

Strain	Phage type	Penicillinase	Reaction to streptomycin (0·5 mg./ml.)
524 sc	—	Inducible	S*
U–9	80/81†	Inducible	R
8510	6/7/47/53/54/75/77‡	Negative	—
8511	53/54/75/77‡	Inducible	—
8325	47/75	Negative	S
8325–18	29/47/75	Inducible	S
S–1	29/47/53/75/77	Inducible	R
Y–4	—	Inducible	R

* S, sensitive; R, resistant.
† According to Pattee & Baldwin (1961).
‡ According to Central Public Health Laboratories, Colindale, London, N.W. 9.

pigmented mutant of s-1 which occurred spontaneously. It should be emphasized that a heterogenetic strain cannot be assumed to be genetically equivalent to a wild type; the interpretation of the results to be reported is therefore of limited generality pending the examination of possible differences between the two.

Growth experiments. Opacity, dry weight and viable count were determined for a suspension of 8325 harvested during exponential growth, centrifuged and resuspended in 0·15 M-sodium chloride. An opacity of 0·10 on the Hilger 'Spekker' absorptiometer, with an Ilford filter No. 508, corresponded to 0·022 mg. bacterial dry wt./ml. This bacterial concentration usually gave about 4×10^7 colony-forming units/ml., corresponding to about 10^8 organisms/ml., since the staphylococci usually grew in clumps of 2–4.

For penicillinase induction tests, strains were grown overnight on 0·5 CY slopes and inoculated into prewarmed CY medium containing μM 2,6-dimethoxyphenylpenicillin (methicillin) to give a suspension equivalent to 0·02–0·04 mg. dry wt. bacteria/ml. The cultures were then shaken at 35° and at the end of 5 hr. were chilled in ice and their opacities measured. Uninduced cultures for penicillinase measurement were identical except that methicillin was omitted.

Bacteriophage strains. The phages of the International Typing Series were obtained from the Central Public Health Laboratories, Colindale, London, N.W. 9. A few single plaques were obtained when phages 53 and 80 were plated on strains 524 sc and 8325, respectively, using 10^8 plaque-forming units/plate. The phage strains thus isolated were designated as 53α and 80α; it is not known whether they are

host-range mutants or host-induced phenotypic modifications. Phage 47′ is a temperate phage carried by strain 8325 in the prophage state.

Propagation of phages was done in 0·3 CY broth or by the soft agar layer method of Swanstrom & Adams (1951) with 0·3 CY medium plates. Temperate phage lysates were obtained by ultraviolet (u.v.) irradiation, with a u.v. dose of radiation which gave 90–95% survival of a non-lysogenic derivative of the same strain. Bacteria were irradiated in 0·15 M-sodium chloride and were then incubated in 0·3 CY broth with gentle shaking at 35° until lysis was complete (usually about 3 hr.). Phage lysates were clarified by centrifugation and bacteria removed by membrane filtration. Phage concentrates free from growth medium for use in u.v.-irradiation experiments were prepared by centrifugation for 75 min. at 35,000 *g* in the Spinco model L preparative centrifuge. The bacterial strains used as indicators for the titration of the phages were 8511 for phage 53, U-9 for phage 80, 8325 for phages 53α and 80α, and 8510 for phage 47′.

Transduction experiments. Recipient organisms were grown overnight on 0·5 CY slopes and were inoculated into 0·5 CY medium to give equiv. 0·2–0·4 mg. dry wt. bacteria/ml. These cultures were shaken at 35° until they had grown to a density of about equiv. 1·0 mg. dry wt./ml. and were then mixed with an equal volume of phage in phage buffer. Calcium chloride was added to a final concentration of 4 mM. For non-lysogenizing phage, multiplicities of infection were 0·3–1·0; for temperate phage, 1·0–10. After further incubation for 45 min. with gentle rocking at 35°, the cocci were washed by centrifugation or membrane filtration to remove free penicillinase and were then plated on appropriate media in soft agar (0·5 %, w/v) overlays. In some experiments they were plated directly on selective medium containing benzylpenicillin (0·06–0·12 μg./ml.). In other experiments they were plated in soft agar layers on the surface of cellophan or cellulose acetate membranes (Oxoid) and incubated for 2 hr. at 35° on non-selective medium before being transferred to plates containing penicillin. After a further incubation for 18–24 hr. at 35°, penicillinase-positive colonies were usually distinguishable by their haloes of penicillin-sensitive satellites. The transductants were always confirmed on the plates by one of the definitive penicillinase-detection methods and in some cases were scored for unselected markers and tested for penicillinase inducibility.

Detection and measurement of penicillinase

Measurement. Penicillinase activities are expressed in units as defined by Pollock & Torriani (1953; one unit = 1 μmole penicillin hydrolysed/ml./hr. at 30°), with the exception that pH 5·8 was used instead of pH 7·0 since staphylococcal penicillinase is optimally active at pH 5·8 (Novick, 1962*a*).

For activities greater than 0·5 units/ml., penicillinase was assayed by the method of Perret (1954). This method could be used with whole cultures, washed cocci, or culture fluids. For measurement of lower penicillinase activities, the following modification, by Dr D. Dubnau (unpublished), of the micro-iodometric method of Novick (1962*b*) was used. The cocci to be assayed were centrifuged and resuspended in 0·15 M sodium chloride to give a maximum suspension concentration equiv. 10 mg. dry wt. bacteria/ml. A dilution was made for opacity determination, and a 1 ml. sample containing from 0·010 to 0·10 units of penicillinase activity was pipetted into a plastic centrifuge tube at 30° containing 1 ml. 0·2 mM-sodium

benzylpenicillin in 0·1 M-sodium phosphate buffer (pH 5·8) with 0·15 % (w/v) gelatin. After incubation for 1 hr. at 30°, 1 ml. of 0·15 M-sodium tungstate in 2·0 M-sodium acetate buffer (pH 4·0) was added to stop the reaction (Csányi, 1961). Each sample had a control in which substrate alone was incubated, enzyme being added after adding the tungstate. Tubes were then centrifuged at 3000 rev./min. for 10 min., decanted, and 2 ml. of supernatant fluid added to 2 ml. of starch iodide reagent. The starch iodide reagent (Novick, 1962*b*) contained 0·4 % (w/v) starch with 80 μM-iodine and 1·6 mM-potassium iodide. After 10 min. at room temperature (about 18°) each sample was read against its own control in 0·5 cm. cuvettes in a Spekker absorptiometer, with an Ilford filter No. 607. The assay gave extinctions that were a linear function of enzyme activity. Readings were converted to enzyme activities with the aid of a standard curve obtained with an enzyme preparation of known activity. The lower limit of sensitivity of the assay was 0·001 units penicillinase/mg. dry wt. bacteria. The advantage of this modification lay in stopping the reaction; thus many samples could be run simultaneously.

Detection. Penicillinase activity was detected in colonies growing on plates by two techniques. The starch iodide test of Perret (1954) gave a positive result with strains that had as little activity as 0·05 units penicillinase/mg. dry wt. bacteria. This method was used to screen suspected penicillinase-negative mutants and to identify penicillinase-positive recombinants on membranes. The Andrade indicator method (Kogut *et al.* 1956) gave a positive reaction only with colonies that had at least 50 units penicillinase activity/mg. dry wt. bacteria. Constitutive mutants and induced wild-type strains gave a positive Andrade test, while uninduced wild-type strains were negative. Thus the method was useful for testing suspected constitutive mutants and for testing penicillinase-positive transductants for inducibility.

Mutations involving penicillinase formation

Induction with ethylmethane sulphonate (EMS). All of the mutations studied in this investigation were induced by EMS according to the procedure of Loveless & Howarth (1959). Strains to be treated were prepared as for transduction experiments. Two ml. of whole culture were added to 0·08 ml. of EMS which was dissolved by gentle pipetting. The bacterial suspension was incubated at 35° for 20 min., 8 ml. of 0·15 M-sodium chloride added, the bacteria centrifuged down, then resuspended in 10 ml. 0·5 CY broth and incubated for 4 hr. at 35°, with shaking before plating.

Isolation of mutants. A method was developed for the isolation of penicillinase-negative and penicillinase-constitutive organisms. This was based on a procedure in which a population of colonies was first grown on a membrane and then transferred to a penicillinase-indicating medium (Knox & Smith, 1961). In the present method, the reason for the transfer step is that the indicator was growth-inhibitory and thus could not be incorporated into the growth medium. To screen large numbers of organisms, 30 × 30 cm. glass trays (Jencons, Ltd., Hemel Hempstead, Hertfordshire, England) were used for growing the colonies. To avoid acid formation during growth, glucose was omitted. A cellophan membrane on each agar surface was spray-inoculated with 10^4–10^5 colony-forming units suspended in 5 ml. soft (0·5 %, w/v) agar. This was followed by a further 5 ml. of soft agar to prevent spreading of the colonies. The trays were incubated overnight and were dried for

2 hr. at 35° with lids off. The membranes were then transferred to acid-base indicator agar and after further incubation (usually 2–4 hr.), three different phenotypes were readily distinguishable: (i) wild-type inducibles had small intense purple haloes, (ii) constitutives had large ones, (iii) penicillinase-negative colonies were white on a reddish background. To recover negatives it was necessary to subculture on agar containing penicillinase (2 units/plate). Thus far 55 penicillinase-negative and 20 penicillinase-constitutive mutants have been isolated.

Table 3. *Classification of mutants of* Staphylococcus aureus

Penicillinase phenotype	u*	Induction ratio† (i/u)	Class designation
Wild-type, inducible	~ 10	~ 30	*pen-rw*
Wild-type, penicillinase-negative	< 0·001	—	*pen-sw*
Loss mutants sensitive‡ to penicillin	< 0·001	—	*pen-sd*
Constitutive	> 10	< 5	*pen-c*
Microconstitutive	< 10	< 5	*pen-mc*
Mesoinducible	0·1–10	> 5	*pen-mei*
Microinducible	< 0·1	> 5	*pen-mi*

* Enzyme concentration in units/mg. dry wt. bacteria in whole cultures in absence of inducer.
† Ratio of enzyme concentration of fully induced culture to concentration in culture grown in absence of inducer.
‡ Sensitive is used to signify that there was no demonstrable effect of inoculum size on minimal inhibitory concentration of penicillin.

Nomenclature and classification of mutants. Penicillinase activities were measured for most of the mutants, both those induced with methicillin and those uninduced. Unexpectedly, many of the supposed penicillinase-negative strains turned out to have appreciable amounts of penicillinase ranging from barely detectable amounts to just lower than the wild-type values, and with induction ratios varying from 1 to 50. It was found convenient to classify the mutants phenotypically. The nomenclature is based on that adopted by Pollock (1957), the various classes having been delimited arbitrarily as shown in Table 3. Thus, all mutants with induction ratios smaller than 5 were classified as constitutive; in this group, those with uninduced penicillinase activities lower than that of uninduced wild type were termed microconstitutive. Among the mutants with induction ratios larger than 5, the inducible class, those with uninduced penicillinase values below 0·1 unit penicillinase/mg., have been termed microinducible, while those which were between that value and the uninduced wild-type value were designated mesoinducible. A final class, mutants with no detectable penicillinase activity, have been termed *pen-sd* mutants.

RESULTS

Penicillinase levels of mutants. In Table 4 are listed the penicillinase values obtained for a representative sample of the mutants. The penicillinase activities for strains with uninduced values below 0·1 unit/mg. dry wt. organism were measured by the micro-assay with washed cocci. All the others were measured by the iodometric assay (Perret, 1954) with whole cultures. Since staphylococcal penicillinase is generally 40–60 % extracellular (Novick, 1962*a*) in mutants as well as in wild-

Table 4. *Mutations in* Staphylococcus aureus *affecting penicillinase formation*

See text for details.

Strain	Phenotype	Penicillinase, units/mg. dry wt. bacteria		Induction ratio (i/u)	Approximate reversion frequency	Mutagen used to induce reversions
		Uninduced (u)	Induced (i)			
8325	Wild-type negative	—	< 0·001	—	$< 2 \times 10^{-11}$	EMS*
8325–18	Wild-type inducible	10	300	30	—	—
pen-sd 7	Negative variant	—	< 0·001	—	$< 2 \times 10^{-11}$	EMS
pen-sd 16		—	< 0·001	—	$< 10^{-10}$	EMS
pen-sd 21		—	< 0·001	—	$< 10^{-9}$	EMS
pen-mi 1	Microinducible	0·0023	0·083	36	—	—
pen-mi 15		0·0058	0·046	8	$\sim 10^{-8}$	u.v.
pen-mi 23		0·0061	0·14	23	$\sim 10^{-8}$	u.v.
pen-mi 36		0·017	0·61	37	—	—
pen-mei 50	Mesoinducible	0·11	1·32	12	—	—
pen-mei 2		0·40	17·3	41	—	—
pen-mei 38		1·08	20·0	19	—	—
pen-mei 52		3·16	121	38	—	—
pen-mc 39	Microconstitutive	0·021	0·018	0·8	—	—
pen-mc 29		0·048	0·039	0·8	$\sim 10^{-6}$	Spontaneous
pen-mc 45		0·60	1·23	2·0	—	—
pen-mc 47		6·60	8·95	1·4	—	—
pen-c 32	Constitutive	28·2	36·4	1·3	—	—
pen-c 25		39·7	74·2	1·9	—	—
pen-c 19		46·4	193	4·2	—	—
pen-c 23		347	327	0·9	—	—
pen-c 27		486	495	0·9	—	—
pen-c 1		643	630	1·0	$< 10^{-4}$	EMS

* EMS, ethylmethane sulphonate.

type strains, measurements carried out with washed cocci are too low by a factor of about 2. Table 4 shows that in both the inducible and constitutive groups there was a continuous range of penicillinase values.

Mutation frequencies. In Table 5 are noted the approximate frequencies of occurrence of the three major classes of mutants, both spontaneous and after EMS treatment. Mutation to streptomycin resistance (0·5 mg./ml.) is included for comparison. Table 5 shows (1) that EMS, while increasing the yield of all mutants with residual penicillinase activity by 10^2–10^3-fold, appeared to have no effect on the incidence of *pen-sd* strains; (2) that the frequency of *pen-sd* was about 10^{-3} (this has been found for other strains also, as will be seen in a later section); (3) that the frequency of EMS-induced mutations involving penicillinase in this strain is higher than it is for streptomycin resistance and for various markers in other organisms (Loveless & Howarth, 1959; Strauss, 1962).

Table 5. *Approximate frequencies of occurrence of various mutant types of* Staphylococcus aureus

S. aureus strain 8325–18 was plated on cellophan membranes on 0·5 CY agar without glucose to give 10^5–10^6 colonies on each membrane. The cocci were either plated directly or were treated with ethylmethane sulphonate (EMS) and then incubated for 4 hr. in 0·5 CY broth before plating. After overnight incubation, the membranes were transferred to acid-base indicator agar and the various types were picked and scored. For streptomycin resistance, 10^9 cocci were plated on 0·5 CY agar with streptomycin (0·5 mg./ml.) both untreated and following EMS treatment and incubation.

Penicillinase phenotype	Frequency	
	Spontaneous	EMS-treated
Constitutive	10^{-6}	5×10^{-4}
Sensitive	10^{-3}	10^{-3}
Microinducible + mesoinducible + microconstitutive	10^{-5}	5×10^{-4}
Streptomycin resistant	5×10^{-9}	3×10^{-8}

Reversions. Several of the mutants were tested for reversion to wild type with regard to penicillinase production. In some cases, cultures were treated with EMS and allowed 6–18 hr. of growth in non-selective medium before plating. Alternatively, cultures were u.v.-irradiated to 1 % survival, allowed to grow overnight in non-selective medium, and then plated on penicillin for selection of revertants; the results are shown in Table 4. No revertants were obtained either spontaneously, with u.v.-radiation, or with EMS treatment from any of the *pen-sd* strains or from strain 8325. Of three strains tested that had detectable penicillinase activity, all gave revertants. The results with one strain, *pen-mc* 29, were interesting in that of 130 revertants, 100 gave positive Andrade reactions, indicating that they were high-level constitutives. The other 30 gave negative Andrade tests, both with and without the inclusion of methicillin in the agar; these might have been microconstitutives or microinducibles, but not wild-type inducibles. Strains *pen-mi* 15 and *pen-mi* 23, on the other hand, both gave rise to revertants which were mostly wild-type inducible by the Andrade test. None of the revertants has yet been tested further.

Genetic analysis

Phenotypic lag. A transduction experiment was carried out in which phage 47′ prepared from strain 8325–18 was used to infect strain 8325. After absorption of

phage, the recipient cocci were plated on Oxoid membranes on 0·5 CY medium, incubated for 2 hr., then transferred to 0·5 CY medium with 0·08 μg. penicillin/ml. This 2 hr. incubation period resulted in a fourfold increase in total transductants over a control plated directly on penicillin-containing agar. It has not been established whether this effect was the result of delayed expression of the newly received penicillinase gene or was due simply to the growth of penicillinase-positive transductants into more resistant microclones during the 2-hr. period before challenge. When the technique of incubation on non-selective medium before challenge with penicillin was used, transduction frequencies of about 5×10^{-5}/ plaque-forming unit were obtained with phage 47′.

Recombination between pen-sd *and microinducible strains.* To explore further the genetic basis of the *pen-sd* mutants and the wild-type penicillinase-negatives (*pen-sw*), and also to begin mapping the penicillinase region, various recombination experiments were carried out as listed in Table 6. Phage 47′ at a multiplicity of 10 was used for most of the crosses; phage 80α at a multiplicity of 1·0 was used for a few. In most experiments, the selective penicillin concentration was 0·09 μg./ml.; the cocci were usually pre-incubated on membranes for 2 hr. before transfer to penicillin. Recombinants were identified on starch iodide indicator agar and were confirmed by scoring unselected markers.

Table 6. *Recombinations between* pen-sd, pen-sw, *microinducible and microconstitutive strains of* Staphylococcus aureus

See text for details.

Donor	Recipient	Phage input	m.o.i.*	Recombinants	
				Inducible	Con-stitutive
pen-mi 1	*pen-mi* 15	10^9	10	150	0
pen-mc 29	*pen-mi* 15	10^9	10	100	0
pen-mc 29	*pen-mi* 1	10^9	10	30	0
pen-mc 29	*pen-sw*†	10^{10}	10	0	0
pen-mc 29	*pen-sd* 16	10^{10}	10	0	0
pen-mi 1	*pen-sd* 16	10^{10}	10	0	0
pen-mi 15	*pen-sd* 7	10^9	1	0	0
pen-sd 7	each of 7 *pen-sd* strains	10^9	1	0	0

* m.o.i., multiplicity of infection.
† the *pen-sw* strain used was 8325

The results given in Table 6 show that when any *pen-sd* or *pen-sw* strain was used either as donor or as recipient in a cross, no wild-type recombinant was observed. When two microinducible strains were crossed, wild-type recombinants were obtained in all of the combinations tried. This was also true for crosses in which the micro-constitutive, *pen-mc* 29, was used as donor. No polarity was observed in reciprocal crosses.

Linkage of structural and control loci. Although *pen-sd* and *pen-sw* strains did not give recombinants among one another or with microinducible strains, it was nevertheless possible that they might have the locus responsible for the inducibility of the enzyme. To test for this, several crosses were done, the results of which are given in Table 7. These crosses were scored for transductants having the donor phenotype and for novel phenotypes which might have arisen by recombination within the

penicillinase region. In one cross, with a constitutive strain *pen-c* 1 as donor and a microinducible strain *pen-mi* 15 as recipient, wild-type inducible recombinants occurred at a frequency of about 0·6 % of the total transductants. Thus, the sites of these two mutations are tightly but not inseparably linked. In two other crosses with the same donor, but with *pen-sd* or *pen-sw* strains as recipients, no such recombinants were observed; all the transductants had the constitutive phenotype of the donor.

Table 7. *Linkage of structural and control loci for penicillinase in* Staphylococcus aureus

Transducing phage was mixed with recipient staphylococci and incubated for 45 min. The coccal suspensions were then membrane filtered or centrifuged to remove penicillinase and unadsorbed phage and were plated on cellophan membranes on 0·5 CY agar without glucose, with 0·12 μg. benzylpenicillin/ml. Transductants were scored on indicator agar after overnight growth and several colonies of each class were tested on Andrade plates for confirmation of phenotype.

Donor	Recipient	Phage input	m.o.i.*	Recombinants	
				Inducible	Constitutive
8325–18	*pen-sw*†	10^9	1	25,000	0
pen-c 1	*pen-sw*†	10^9	1	0	8000
pen-c 1	*pen-sd* 7	10^9	1	0	3000
pen-c 1	*pen-mi* 15	10^8	10	3	500

* m.o.i., multiplicity of infection.
† the *pen-sw* strain used was 8325.

Clearly, the *pen-sd* mutants are not allelic to *pen-c* 1, so that in failing to give wild-type recombinants in crosses with the latter, they behaved as though they lack the regulatory locus which carries the mutation of *pen-c* 1. The possibility that the *pen-sd* strains tested are double mutants carrying a lesion allelic to *pen-c* 1 as well as another, is made unlikely by the failure to obtain constitutive recombinants in crosses with the wild-type inducible strain 8325–18.

Effect of ultraviolet-irradiation. Figure 1 shows the results of an experiment in which a suspension of phage 80α was u.v.-irradiated and serial determinations of transducing and plaque-forming titres carried out. As can be seen from Fig. 1, there was exponential inactivation of transducing as well as of plaque-forming activity as a function of dose of u.v. radiation. It has been observed previously that exponential u.v. inactivation is associated with transduction of genetic determinants that are not integrated into the recipient chromosome. On the other hand, u.v.-irradiation, in low doses, has been observed to increase the frequency of stable recombinants for markers that apparently are integrated (Fig. 1, broken curve), (Garen & Zinder, 1955; Arber, 1960; Korman & Berman, 1962).

Effect of acridine orange. Ultraviolet-irradiation (Bondi *et al.* 1953) and EMS treatment did not increase the frequency of *pen-sd* variants, nor did growth in the presence of acridine orange. Table 8 shows the results of experiments in which single colonies of several strains were picked and grown in the presence of acridine orange according to the procedure of Hirota (1960). For testing, the cultures were plated to give well-separated colonies which were assayed by the starch iodide test for penicillinase (Perret, 1954). As shown in Table 8, the frequencies of penicillinase-negative colonies were similar for all of the four strains tested whether growth had taken place with or without acridine orange.

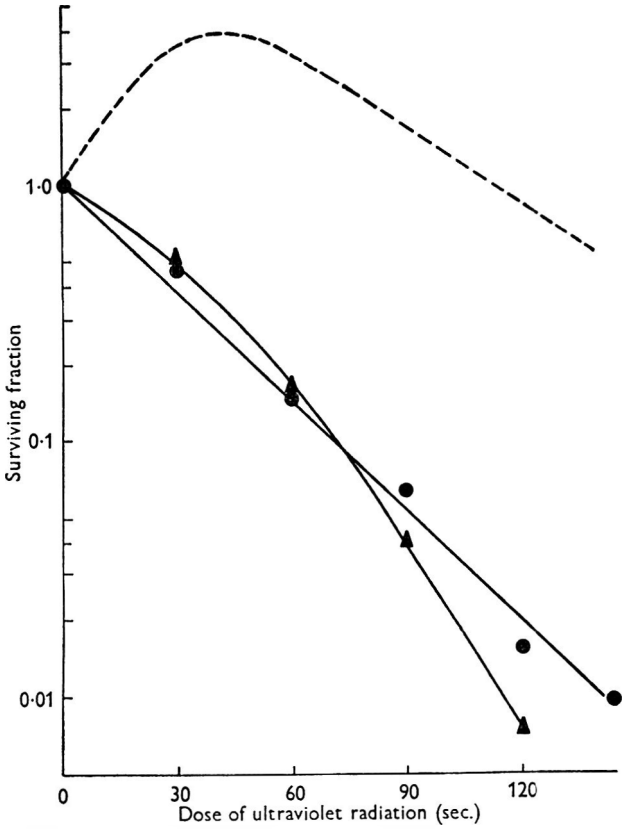

Fig. 1. The effect of ultraviolet-irradiation on a penicillinase-transducing phage lysate. Phage 80 α, propagated on *Staphylococcus aureus* 8325–18, was diluted in phage buffer to give 10^{10} plaque-forming units/ml. and was u.v.-irradiated for various lengths of time. Plaque-forming titre (▲) and transducing titre for penicillinase (●) were determined for each dose of u.v. radiation and are plotted semilogarithmically. The broken curve illustrates the effect of u.v. radiation on transduction of chromosomal markers in *Escherichia coli* (Garen & Zinder, 1955; Arber, 1960).

Table 8. *Loss of penicillinase in wild-type strains of* Staphylococcus aureus; *effect of acridine orange*

		Control cultures		Acridine orange cultures†	
Strain	Growth cycles*	Total colonies	*Pen-sd* colonies	Total colonies	*Pen-sd* colonies
Y–4	SC‡	3030	3	—	—
Y–4	2	4320	8	4300	3
U–9	SC	3229	6	—	—
U–9	2	5370	16	7800	15
U–9	3	—	—	1142	5
524	3	—	—	2850	3
8325–18	3	—	—	4355	7

* Number of 18 hr. subcultures inoculated with 10^4 organisms/ml.

† Acridine orange concentration used with strain Y–4 was 5 μg./ml.; with all others it was 10 μg./ml.

‡ SC signifies single colony picked and plated directly.

DISCUSSION

The mutants of *Staphylococcus aureus* which have been isolated and examined thus far appear to fall into at least three distinct categories: the constitutive group, the inducibles, and the *pen-sd* strains. The subdivisions within those categories that have been made are arbitrary and were introduced simply for convenience.

Constitutive mutants

Phenotypically, the constitutive and microconstitutive mutants have alterations in the system which regulates the induction of penicillinase by penicillin. It remains to be seen whether, by analogy with the β-galactosidase of *Escherichia coli* (Jacob & Monod, 1961) or the alkaline phosphatase of the same organism (Garen & Echols, 1962), there is more than one distinct locus in the regulatory system for penicillinase. It will also be important to work out the dominance relationships between the wild type and its mutant alleles in whatever regulatory loci are found. In any case, if the regulatory locus or loci are distinct from the structural locus, the constitutive mutants should form normal wild-type penicillinase at specific rates which are characteristic of the strains.

Inducible mutants

The specific genetic alterations responsible for most of the microinducible and mesoinducible mutants have yet to be identified; some information, however, is available. Those genetic alterations thus far examined appear to be point mutants; at least two, both mesoinducible, appear to have structural alterations in the penicillinase molecule; Dr M. H. Richmond (personal communication) has found that the penicillinases produced by these react atypically with anti-penicillinase serum. On the other hand, Dr Richmond has found that others of the inducible group produce penicillinases which react normally with antiserum. The latter might represent mutations involving (1) stability of the enzyme, (2) rate of production and molecular structure, (3) control of enzyme formation, (4) accessibility of enzyme to substrate (cryptic mutants), or (5) antigenic specificity as well as enzymic activity. These possibilities have not yet been examined experimentally, but among them crypticity is considered to be unlikely. This is because many of the mutants have inducibility ratios similar to the wild type as well as lower resistance to penicillin. Crypticity would thus entail the existence of intracellular compartments such that penicillin could penetrate to the site of enzyme induction as well as to the site of its antimicrobial action, while at the same time being unable to reach the sequestered enzyme.

Penicillinase-negative strains

The attempts at the genetic characterization of the *pen-sd* and the wild-type penicillinase-negative strains have given rise to the hypothesis of an extrachromosomal mode of inheritance for penicillinase production in *Staphylococcus aureus*. Under this hypothesis, the extrachromosomal particle involved would simply be missing from the *pen-sd* and *pen-sw* strains. Since some of the experiments were undertaken with such a possibility in mind, it is appropriate to review the evidence obtained.

Evidence in favour of extrachromosomal inheritance. (1) The capacity to produce

penicillinase is frequently lost by individual cocci of most of the strains of Staphylococcus studied here and by other workers (Barber, 1949; Bondi *et al.* 1953; Fairbrother, 1956). This occurrence is probably too frequent to be accounted for by a chromosomal deletion. Further, its incidence is not increased by mutagens such as ethylmethane sulphonate or ultraviolet radiation. (2) The *pen-sd* variants of penicillinase-positive strains and the *pen-sw* strains apparently do not revert to penicillinase production. (3) The *pen-sd* and *pen-sw* strains have not been observed to give penicillinase-positive recombinants when crossed to strains with documented point-mutations in the penicillinase region; nor have they been shown to possess the locus which controls inducibility of the enzyme. (4) Ultraviolet-irradiation of phage produced exponential loss of penicillinase-transducing activity; transduction of other staphylococcal markers was found by Korman & Berman (1962) to be stimulated by u.v.-irradiation of transducing phage. As pointed out in Results, exponential u.v.-inactivation is characteristic of transduction of genetic particles which do not become integrated into the recipient chromosome. Among the classes of such particles previously studied, all in coliform organisms, are certain of the episomes (Arber, 1960) and chromosomal fragments carried by defective lysogens (Luria *et al.* 1960). Of these, the latter does not appear to be the situation here because all of 100 transductants tested were found to be sensitive to the transducing phage. If, indeed, an extrachromosomal particle is involved in penicillinase inheritance, in the absence of evidence regarding reversible attachment to the chromosome, the term 'plasmid' (Lederberg, 1952) will be preferable to 'episome' (Jacob & Wollman, 1958) in describing it. It should be emphasized that the above interpretation of exponential u.v.-inactivation depends on an extrapolation from coliform organisms to staphylococci; the validity of this has still to be tested.

Evidence against extrachromosomal inheritance. No increase in *pen-sd* mutants resulted from growth in the presence of acridine orange. This compound and other acridine dyes have been found to promote the loss of various plasmids and episomes (Ephrussi, Hottinguer & Chimenes, 1949; Hirota, 1960; Watanabe & Fukasawa, 1961), but this has not been found for all such particles (Hirota, 1958). Thus the present results with penicillinase are not necessarily incompatible with extrachromosomal inheritance.

At the present time, there is more evidence in favour of the plasmid hypothesis for penicillinase inheritance in *Staphylococcus aureus* than against it. However, much of the work reported here is of a preliminary nature and considerably more needs to be done before the question can be settled. Besides further experiments along lines already outlined here, it will be important to determine whether any linkage relationships exist between penicillinase and other markers, and whether the penicillinase region can, like the episomes, be transferred by cell-to-cell contact (Jacob, Schaeffer & Wollman, 1961).

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The Production of Riboflavin and D-Arabitol by *Debaryomyces subglobosus*

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SUMMARY

Debaryomyces subglobosus (British National Collection of Yeast Cultures, NCYC 459) excretes riboflavin at the end of the growth period in batch culture and the yield depends on the concentration of iron in the medium. Yields of riboflavin in excess of 200 μg./ml. were obtained in media of low iron content but production was completely inhibited at Fe concentrations greater than 200 μg./l. D-Arabitol was produced simultaneously, increased yields of it being obtained at high iron concentration, but riboflavin production was then decreased. Procedures are described for the recovery of riboflavin and D-arabitol from the culture fluid.

INTRODUCTION

Riboflavin is produced to some degree by a variety of micro-organisms (see, for example, Pridham, 1952). Outstanding in this respect are the two Ascomycetes, *Eremothecium ashbyii* (Guilliermond, Fontaine & Raffy, 1935; Raffy, 1937) and *Ashbya gossypii* (Wickerham, Flickinger & Johnson, 1946; Tanner, Wickerham & Van Lanen, 1948). These fungi are capable of giving yields of riboflavin in the region of 2000 μg./ml. under appropriate conditions (Moss & Klein, 1947; Pridham, 1951). Significant amounts of riboflavin are also yielded by certain clostridia (e.g. Meade, Rodgers & Pollard, 1947) and by some yeasts including, mainly, species of the genus *Candida* (Burkholder, 1943; Tanner, Vojnovich & Van Lanen, 1945) and a *Torulopsis* sp. (Dikanskaya, 1962). Unlike the fungal processes, production of riboflavin by clostridia and yeasts is generally subject to inhibition by iron; for example, Tanner *et al.* (1945) found the optimal concentration of iron for *Candida guilliermondii* was 5–10 μg./l. and that iron concentrations of 100 μg./l. sharply decreased the yield of riboflavin. With this yeast Enari & Kauppinen (1961) showed that the inhibition of riboflavin production by iron could be annulled to some extent by adding cobalt to the medium.

Since the first observation of the production of D-arabitol by members of the genus *Zygosaccharomyces* (Spencer & Sallans, 1956) it has been reported that other yeasts give rise to this and related substances (e.g. Onishi, 1960). Fermentation procedures have been worked out for the production of D-arabitol and yields of as much as 45 % by weight based on the sugar content of the medium have been obtained (Lavin & Holloway, 1960).

The present paper describes the conditions for producing riboflavin and D-arabitol by the yeast *Debaryomyces subglobosus*.

METHODS

Organism. A strain of *Debaryomyces subglobosus* (NCYC 459) was used except where otherwise indicated. It was maintained on a medium containing (g./l.): malt extract, 3; yeast extract, 3; peptone, 5; glucose, 10; agar, 20 (Wickerham, 1951).

Culture media. The defined medium used for the production or riboflavin and D-arabitol contained salts and vitamins as formulated by Wickerham (1951). Trace elements were added as H_3BO_3, $MnSO_4.H_2O$, $CuSO_4.5H_2O$, $Na_2MoO_4.2H_2O$, $ZnSO_4.7H_2O$ and KI to give final concentrations of boron, manganese, copper and molybdenum (20 μg. each/l.), zinc (140 μg./l.) and potassium iodide (100 μg./l.). Glucose in various concentrations and ammonium sulphate (0·5 %, w/v), except where otherwise stated, provided the sole sources of carbon and nitrogen, respectively. Iron was added as $FeCl_3.6H_2O$ and cobalt as $CoCl_2.6H_2O$.

Solutions of glucose, of ammonium sulphate + salts, and of trace elements were autoclaved at 120° for 15 min. Solutions of the vitamins were sterilized by Seitz filtration.

The cultures, contained in Roux bottles or medical bottles, plugged with cotton wool, were shaken at 25°.

Riboflavin assay. The riboflavin excreted into the medium was estimated by optical density measurements of cell-free broth at 375 and 445 mμ and a calibration curve prepared by using pure riboflavin.

Chromatography: qualitative. Descending paper chromatograms were prepared at room temperature on Whatman No. 1 paper with the following solvents: (*A*) butanol + acetic acid + water (4 + 1 + 1, by vol.; Hais & Pecàkovà, 1949); (*B*) propanol + ethyl acetate + water (7 + 1 + 2, by vol.; Gross & Albon, 1953); (*C*) methylethylketone + acetic acid + water (9 + 1 + 1, by vol.; saturated with boric acid; Rees & Reynolds, 1958).

Silver nitrate + sodium hydroxide (Trevelyan, Proctor & Harrison, 1950), or periodate + benzidine (Cifonelli & Smith, 1954), was used as the spray reagent.

Chromatography: preparative. The preparative separation of D-arabitol from glucose was achieved by descending chromatography at room temperature on Whatman No. 3 paper with solvent *B* above. The separated components were eluted from the paper with water.

Removal and estimation of iron. Glucose was freed from iron by the passage of an aqueous solution of the sugar (6 %, w/v) through a column containing excess Zeokarb 225 cation-exchange resin which had been converted to the H^+ form by treatment with 2 N-HCl followed by the removal of the excess acid by washing with distilled water. The glucose concentration of the eluate was determined polarimetrically. Whole medium, less trace elements and vitamins, was in certain cases freed from iron by treatment with 8-hydroxyquinoline (2 % in chloroform) as is described in Results. Iron was estimated in concentrated medium by the *o*-phenanthroline method (Bandemer & Schaible, 1944).

Recovery of riboflavin and D-arabitol from the culture fluids. All evaporations were carried out at 20° under reduced pressure.

The culture fluid, after removal of the organism by centrifugation, was evaporated to small volume and ethanol added to 70 % (v/v). After removal of the resultant precipitate, the solution was evaporated to small volume and passed on to a column

of Dowex 50 cation-exchange resin (H^+ form); the riboflavin was retained at the top. Neutral and acidic materials were first eluted from the column with water anp the riboflavin was then eluted with dilute ammonium hydroxide solution. The ammoniacal solution was evaporated to dryness and the resultant yellow residue was crystallized and recrystallized from dilute acetic acid to give a product which proved identical with an authentic sample of riboflavin.

The aqueous eluate from the Dowex 50 column was evaporated to small volume and the solution was then passed on a column of Dowex 1 anion-exchange resin previously converted to the acetate form by successive treatment with 2N-NaOH, water and 2M-sodium acetate and finally washing with water until neutral. The neutral components of the culture fluid were eluted from the column with water and this eluate evaporated to dryness. In those cases when the bulk of the glucose had been utilized by the yeast, the D-arabitol was readily obtained in pure form from the residue by crystallization and recrystallization from ethanol. Where there was an appreciable amount of glucose remaining at the end of the growth, this was first separated from the D-arabitol by chromatography.

RESULTS

It was found that cotton wool plugs in the culture vessels allowed access of sufficient air for the production of riboflavin; more vigorous aeration did not increase the yields. The media were unbuffered and with ammonium sulphate as N-source the pH value decreased from 5·2 (initial) to about 2·6 within 24 hr. after inoculation. The maximum yields of the products were, in the main, produced 5–6 days after inoculation.

Production of riboflavin

Untreated medium. Defined medium (400 ml.) containing glucose (1 %, w/v) was inoculated with 0·1 ml. of a 48 hr. culture of the organism grown in the same medium. At the end of the logarithmic period of growth (after incubation for about 26 hr.), the appearance of yellow pigment in the medium was observed. The absorption spectrum of samples of cell-free culture fluid was identical with that of a dilute solution of authentic riboflavin in defined medium. The identity of the pigment was confirmed by chromatographic analysis in solvent *A*; a single fluorescent spot with the same mobility (R_f 0·30) as that of riboflavin was obtained. Riboflavin production was followed by determiming the extinction at 375 and 445 mμ of samples of cell-free culture fluid withdrawn at intervals from the cultures. A maximum yield of more than 60 μg. riboflavin/ml. was obtained (Fig. 1).

When defined medium containing glucose (4 %, w/v) was similarly inoculated, a much lower yield of riboflavin resulted (about 20 μg./ml.; Fig. 1). There was no significant difference in the amount of growth at the two glucose concentrations used.

The effect of iron. To examine the possibility that the lower yield of riboflavin produced at the higher glucose concentration (4 %, w/v) was due to the introduction of an increased concentration of iron with the sugar, the following media were inoculated with the organism: (*a*) defined medium containing glucose (4 %, w/v, untreated); (*b*) defined medium containing glucose (4 %, w/v) which had been treated previously with Zeokarb 225 cation-exchange resin (H^+ form) to remove

cations. The production of riboflavin in medium (*a*) was similar to that observed above with untreated glucose (4 %, w/v; maximum yield of about 20 μg. riboflavin/ml.), while in medium (*b*) a maximum yield of 120 μg. riboflavin/ml. was obtained (Fig. 1).

An increase in the ammonium sulphate concentration from 0·5 to 1·5 % (w/v) in medium containing untreated glucose (1 %, w/v) decreased the yield of riboflavin from 60 to 25 μg./ml. This again could be accounted for by the increased iron concentration.

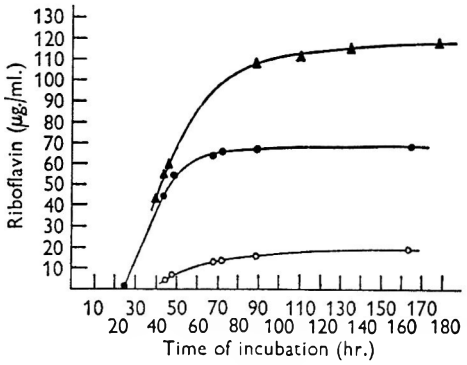

Fig. 1

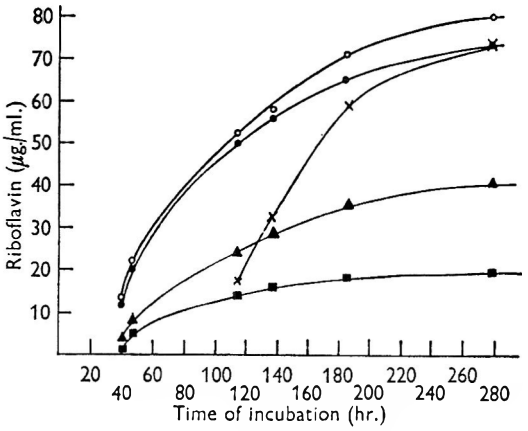

Fig. 2

Fig. 1. Production of riboflavin by *Debaryomyces subglobosus* (NCYC 459) in defined medium containing untreated and treated (de-ferrated) glucose. ●—●, 1 % untreated glucose; ○—○, 4 % untreated glucose; ▲—▲, 4 % glucose treated with Zeokarb 225 cation-exchange resin.

Fig. 2. Effect of iron and cobalt on the production of riboflavin by *Debaryomyces subglobosus* (NCYC 459). The metals were added at the following concentrations to defined medium containing resin-treated glucose (4 %, w/v) and the production of riboflavin was determined. ●—●, No addition; ○—○, iron 20 μg./l.; ▲—▲, iron 40 μg./l.; ■—■, iron 80 μg./l.; ×—×, iron 80 μg./l., cobalt 20 mg./l.

To establish that it was removal of iron and not some other material from the glucose by the resin which resulted in the observed increase in riboflavin production and at the same time to investigate the effect of cobalt on the inhibition of riboflavin production by iron, the following media were inoculated with the organism: defined medium containing 4 % (w/v) glucose (previously treated with resin) and added iron to give final concentrations of 0, 20, 40 and 80 μg. Fe/l., and this last medium + cobalt (20 mg./l.). The course of the production of riboflavin was followed (Fig. 2). In this particular series, the maximum yields of riboflavin were not attained until after a prolonged period of incubation. Iron had a marked inhibitory effect on riboflavin production and the inhibition was decreased by adding cobalt. The presence of cobalt prolonged the lag phase before growth and consequently delayed riboflavin production.

Optimal concentration of iron. The iron concentration of the defined medium which contained untreated glucose (4 %, w/v) was estimated by the *o*-phenanthroline method to be about 100 μg. Fe/l. The medium less trace elements and vitamins was extracted (×4) with equal volumes of a solution of 8-hydroxyquinoline (2 % in

chloroform) and then with chloroform (× 3). Traces of chloroform were removed by passing air through the medium. Little or no iron was detected in concentrates of this treated medium. Trace elements, vitamins and iron at various concentrations, were added to samples (100 ml.) of treated medium which were then inoculated with organism and incubated. Riboflavin production was determined (Table 1). The optimal concentration of iron for riboflavin production was in the range 10 to 20 μg./l. Production of riboflavin was not greatly decreased at concentrations up to 80 μg. Fe/l. (about 30 % inhibition) but there was complete inhibition at 200 μg. Fe/l. and above.

Table 1. *Optimal concentration of iron for the production of riboflavin by* Debaryomyces subglobosus (NCYC 459)

The organism was grown at 25° in defined medium containing glucose (4 %, w/v) and iron at the given concentrations. Riboflavin production was estimated after 7 days.

Iron (μg./l.)	Yield of riboflavin (μg./ml.)
0	60
5	63
10	90
20	95
40	85
80	64
100	56
200	0

The effect on riboflavin production of nitrogen sources other than ammonium sulphate

Corn steep liquor. Corn steep liquor (CSL; kindly supplied by Corn Products Ltd., containing sugars, 2·3 %; nitrogen, 3·9 %; iron, 156 p.p.m.) was diluted 1/4 with distilled water. Iron was removed from a portion of this solution by the treatment with 8-hydroxyquinoline, described above. Media were prepared containing 4 % (w/v) glucose (previously treated with resin), salts, trace elements, vitamins + one of the following N-sources: (i) ammonium sulphate (0·5 %, w/v); (ii) untreated CSL (1/10 dilution); (iii) 8-hydroxyquinoline-treated CSL (1/10 dilution). The media containing CSL were thus of nitrogen contents comparable with the medium containing 0·5 % ammonium sulphate. Riboflavin production was determined in these media. Uninoculated medium containing CSL showed appreciable absorption in the range 320–500 mμ and corrections for this were necessary in the estimation of riboflavin formed in these media. As compared with a yield of riboflavin 90 μg./ml. in medium (i) in 7 days, no riboflavin was produced in medium (ii) and only 56 μg./ml. in medium (iii). CSL therefore presented no advantage in riboflavin production though all the media supported heavy growth.

Amino acids, purines, and pyrimidines. To samples of defined medium (10 ml.) containing resin-treated glucose (4 %, w/v) the following L-amino acids, purines and pyrimidines were separately added to 0·005 M: α-alanine, arginine, asparagine, aspartic acid, cysteine, cystine, glutamic acid, glycine, leucine, lysine, methionine, proline, threonine, valine, adenine, guanine, thymine, uracil, xanthine. The media were inoculated and incubated in the normal way. A significant increase in the production of riboflavin was obtained in the presence, singly, of alanine, arginine,

asparagine, glycine, threonine, valine, adenine, guanine, xanthine, namely, 170–200 μg. riboflavin/ml. as compared with 140 μg./ml. in controls with no supplementary N-source. Cystine and uracil decreased the yield, giving only 86 and 100 μg. riboflavin/ml., respectively. All the N-supplemented media gave an increase in the growth of the organism: 1·4–1·9 mg. dry wt./ml. as compared with 1·2 mg. dry wt./ml. in the controls, with the exception of the cysteine-supplemented medium which gave no growth and no riboflavin.

Glycine. Since glycine promoted riboflavin production, defined medium (400 ml.), containing resin-treated glucose (4 %, w/v), but with glycine (0·5 %, w/v) in place of ammonium sulphate, was inoculated with the organism. Maximum yields of riboflavin of the order of 200–240 μg./ml. were obtained. The pH value of the medium did not follow the same course as when ammonium sulphate was the N-source. In the glycine medium, after an initial decrease to pH 4·4 (at about 20 hr.), there was a gradual increase to about pH 8 at about 120 hr.; the maximum yield of organism was equivalent to 1·8 mg. dry wt./ml.

Riboflavin production by Saccharomyces rouxii. A strain of *Saccharomyces rouxii* (NCYC 561) was grown in the defined medium (100 ml.) containing resin-treated glucose (4 %, w/v) and ammonium sulphate (0·5 %, w/v). Yields of riboflavin of the order of 5–6 μg./ml. were obtained after incubation for 5 days.

Recovery of riboflavin from cultures

The supernatant liquid (400 ml.), after removal of organism by centrifugation of a grown culture (riboflavin content 60 μg./ml.), was processed as described in Methods. A column (10 × 2 cm. diameter) containing Dowex 50 resin (H^+ form) was used. The final product (14 mg., crystallized from dilute acetic acid and dried) had m.p. and mixed m.p. with an authentic sample of riboflavin 270–272°, decomp. (literature gives riboflavin 271°). A solution of the product in water (40 μg./ml.) gave an absorption spectrum identical with that of an aqueous solution of authentic riboflavin (maximum at 220, 268, 375 and 445 mμ). The infra-red spectrum of the product (KBr disk) was identical with that of an authentic sample of riboflavin.

Production of D-*arabitol*

Identification of D-*arabitol in culture fluid.* After removal of organism by centrifugation from a culture in defined medium containing resin-treated glucose (4 %, w/v), the supernatant fluid (riboflavin content 100 μg./ml.) was processed as described in Methods successively with cation- and anion-exchange resins. Chromatographic analysis with propanol + ethyl acetate + water solvent (*B*) of samples of the aqueous eluate from the Dowex 1 resin showed a major component (R_f, 0·43) in addition to glucose (R_f, 0·33) and trace components (R_f, 0·51, 0·64, 0·74) with the silver nitrate + NaOH spray reagent (i). The development of the spot produced by this component was slower than that of reducing sugars. No positive reducing reaction was obtained with the aniline oxalate spray reagent (Horrocks & Manning, 1949). The periodate + benzidine spray reagent (ii) and the bromocresol purple + boric acid reagent of Bradfield & Flood (1950) yielded positive results. These findings suggested that the material was a sugar alcohol. A concentrate of the mixture was run on Whatman No. 3 paper in solvent (*B*) and the strip corresponding to R_f 0·43

was extracted with water. Chromatography of a sample of the resultant solution gave a single spot (R_f, 0·43, with solvent (*B*), spray reagents (i) and (ii)). The remainder of the solution was evaporated to dryness and the solid residue was crystallized from hot ethanol (× 3). The dry product had m.p. 100° and a chromatographic mobility R_f 0·43 in solvent (*B*). Its chromatographic mobility in methylethylketone + acetic acid + water solvent (*C*) was consistent with the substance being either D-arabitol or ribitol (R_f, 0·30 for both; glucose, R_f 0·08). The m.p. of a mixture with authentic D-arabitol was 100° whereas that of a mixture with ribitol (m.p. 100°) was 85°. The infra-red spectrum of the material was identical with that of D-arabitol and differed slightly from that of ribitol. The identity of the product as D-arabitol was thus established.

The course of the production of D-arabitol in iron-deficient medium (4 %, w/v, glucose) was followed by chromatographic analysis of samples withdrawn from the culture at intervals using solvent (*C*), spray reagent (ii). Arabitol was not detected until about 40 hr. after inoculation, and its amount increased, together with riboflavin, over about 5 days. When the amounts of both products had reached a maximum, a considerable amount of glucose remained unchanged.

Production of D-*arabitol at high iron concentration.* The defined medium (400 ml.) containing glucose (4 %, w/v) and iron (1 mg./l.) was inoculated and incubated. D-Arabitol was detected after about 40 hr.; after 5 days no glucose remained and no riboflavin had been produced. At this time the culture fluid (300 ml.) after removal of organism was processed as described in Methods. The aqueous eluate from the Dowex 1 column (20 × 2 cm. diameter) was evaporated to dryness and the residue crystallized from ethanol. Recrystallization from ethanol (× 2) yielded a dry product (1·6 g.) identical with authentic D-arabitol. Increasing the concentration of glucose in the medium to 10 % (w/v) did not result in an increased yield of D-arabitol.

DISCUSSION

Production of riboflavin. Inhibition by iron. In the present work the production of riboflavin by a strain of *Debaryomyces subglobosus* was found to be subject to inhibition by iron, the optimum concentration (10–20 μg. Fe/l.) for riboflavin production being comparable with that found by Tanner *et al.* (1945) for *Candida guilliermondii*. When cobalt was included in a medium containing iron at a concentration which otherwise decreased riboflavin production, the yield of riboflavin was equivalent to that obtained at a lower iron concentration in the absence of cobalt. The cobalt thus appears to compete with iron as suggested by Enari & Kauppinen (1961) in the case of *C. guilliermondii*.

Sources of nitrogen. Several L-amino acids, purines and pyrimidines added singly to the medium as supplementary N-sources did not specifically stimulate riboflavin production. Goodwin & Pendlington (1954), in a similar study on *Eremothecium ashbyii*, found that several similar N-sources stimulated riboflavin production specifically while others also stimulated growth. In the case of the yeast, however, there is the added complication of the inhibition by iron and the results may be better compared with those of Goodwin & McEvoy (1957) who investigated the effect of different N-sources on the iron-sensitive production of riboflavin by *Candida flareri*. These investigators found that replacing ammonium sulphate,

present at a lower level than is used in the present study, either completely or in part by various amino acids, greatly decreased the production of riboflavin.

Glycine was a better N-source for riboflavin production by *Debaryomyces subglobosus* than was ammonium sulphate. Goodwin & McEvoy (1959) showed that glycine as sole N-source, at a concentration comparable with that used here, stimulated riboflavin production by *Candida flareri*. In the present case, however, a comparison between ammonium sulphate and glycine is complicated by the different pH values attained in the medium with the two compounds.

Recovery of riboflavin from the medium. Most of the methods described for the recovery of riboflavin from culture fluids depend on its precipitation in a reduced form. The procedure described here is based on the finding that riboflavin is retained on Dowex 50 resin, the retention apparently resulting from some molecular adsorption rather than from a normal cationic-exchange process since elution cannot be effected by means of dilute mineral acid but is readily achieved by dilute ammonia. It is noteworthy that Zeokarb 225 cation-exchange resin (H^+ form) does not retain riboflavin. Since it is known that riboflavin is degraded under alkaline conditions, the ammoniacal solutions were not exposed to direct sunlight and the ammonia was removed as rapidly as possible.

Production of D-*arabitol.* The present investigation demonstrates that D-arabitol is produced simultaneously with riboflavin by *Debaryomyces subglobosus* in iron-deficient medium. In media of iron content such that the production of riboflavin is completely inhibited, pure D-arabitol amounting to some 13 % of the original glucose present is recovered from the medium by the procedure described.

These findings suggest a possible link between the metabolic pathways involved in the formation of riboflavin and D-arabitol. It has been concluded from studies on the biogenesis of D-arabitol by yeasts (Spencer, Neish, Blackwood & Sallans, 1956; Weimberg, 1962) that the phosphogluconate pathway was involved with transketolase reactions playing an additional role. Weimberg considered that the function of D-arabitol formation may possibly be to regenerate the NADP reduced by the oxidation of glucose-6-phosphate to D-ribulose-5-phosphate. Although it was demonstrated by Weimberg (1962) that the dehydrogenase system in *Saccharomyces mellis* which controls the reaction,

$$\text{D-ribose} \xrightarrow{\text{NADPH}} \text{D-arabitol},$$

had no metal requirements. Onishi & Saito (1962) found that a similar NAD-dependent system from *Pichia miso* was irreversibly denatured in the absence of iron. The situation for *Debaryomyces subglobosus* is not known but if the formation of D-arabitol here is at least in part via a mechanism similar to that put forward by Weimberg, then it may be postulated that the lack of iron results in some inhibition of the dehydrogenase system, by analogy with the findings of Onishi & Saito (1962). The organism may then be considered as synthesizing riboflavin as an alternative hydrogen transport system for the regeneration of NAD or NADP. However, the lack of iron may result in an inhibition of an earlier step in the glucose monophosphate pathway. Thus, Koepsell (1950) found that with *Pseudomonas fluorescens*, when iron was at a low concentration, the oxidation of oxo-gluconate was very slow. It is perhaps significant that in this case a yellow pigment was excreted into the medium at low iron concentration and that the amount of iron necessary for the

stimulation of oxidation of oxo-gluconate was in the range 100–1000 μg./l. which is comparable with the concentrations which bring about the inhibition of riboflavin production by the yeast.

If riboflavin production by *Debaryomyces subglobosus* results from such inhibition then it may be expected that the supply of pentose material for the ribityl side-chain would be restricted to pathways other than that involving oxidation of hexose through oxo-gluconate. A study similar to that of Plaut & Broberg (1956) using a yeast under conditions of iron-deficiency in place of the iron-independent *Ashbya gossypii* may show the different pattern herein suggested for the relative contributions of the pathways involved in the formation of the ribityl side-chain.

It would be expected that other micro-organisms which produce D-arabitol by the phosphogluconate pathway might be induced to produce riboflavin or some such compound in iron-deficient medium. Some support for this is found in the literature, as, for example, Onishi (1960) reported the presence of small amounts of riboflavin in culture fluids of *Pichia miso* (iron content of the medium unstated). Moreover it has been shown in the present study that *Saccharomyces rouxii* (NCYC 561), an osmophilic yeast noted for its arabitol production, does produce riboflavin when the growth medium is deficient in iron.

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Microbial and Biochemical Changes during the Moulding of Hay

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SUMMARY

Experimental batches of hay were baled at different moisture contents, and the microbial and biochemical changes studied by sequential sampling. The type of hay obtained could, in general, be related to the initial moisture content, and to the temperature subsequently attained. Good hays (about 16 % moisture) heated little and contained a small but diverse microflora. Hays baled at about 25 % moisture heated to about 45° and moulded, mainly with *Aspergillus glaucus*. Wet bales, with initial moisture contents of about 40 %, became very hot (60°–65°) and contained a large flora of thermophilic fungi, particularly *Aspergillus fumigatus*, *Absidia* spp., *Mucor pusillus*, *Humicola lanuginosa*, and actinomycetes. During the initial heating period, which was correlated with a general rise in numbers of micro-organisms, particularly actinomycetes and bacteria, the acidity and volatile nitrogen increased. Later, when fungi and actinomycetes grew profusely, soluble sugars decreased rapidly and the pH value rose to 7·0 or above. Stacks of wet and dry hays were compared with bales made from the same hays. The wet stack developed a core of brown acid hay, containing many spore-forming bacteria but few fungi, surrounded by a layer of mouldy hay.

INTRODUCTION

This work was begun to obtain information in connexion with human and animal diseases ascribed to the inhalation of dust from mouldy hay. Hays thought to have caused Farmer's Lung disease contained many thermophilic moulds and actinomycetes (Gregory & Lacey, 1963*a*), but these hay samples were the quiescent end-products of a process, and we needed to know what factors during hay-making produced the various recognizable types of hay. The major factor seemed likely to be the moisture content of the herbage at the time of baling.

In 1959 experiments were started at Rothamsted to follow changes in batches of mainly grass hay baled at different moisture contents; sequential measurements were made of temperature, moisture content, numbers of fungi, actinomycetes and bacteria in seven batches (P.H.G. and M.E.L.). Changes in chemical constituents of stored wet hay have been recorded (Hoffman, 1935; Hoffman & Bradshaw, 1937; Roethe, 1937) but they were not correlated with detailed microbial studies. Accordingly in 1960 sequential samples used for microbial analysis were also studied biochemically (G.N.F.). In 1961 changes in the bacterial population were also studied (F.A.S.). Earlier work on the microbiology of hay has been related to the

problem of self-heating in damp haystacks and the occasional hazard of spontaneous combustion (Miehe, 1930). Bales and stacks of wet and dry hay were compared in 1961. Each batch of hay was studied for 1 season only.

METHODS

Haymaking. The grass used came from non-experimental fields on the Rothamsted farm; in 1959 and 1960 the grass was usually tedded only, but in 1961 it was also crushed twice with a forage harvester. Samples taken frequently during wilting were oven-dried to determine water content so that baling could be timed to give batches at the different required moisture contents. Bales measuring about 42 by 18 by 14 in. were made with a 'B 55' baler (International Harvester Co.), and stored under cover.

Studies on bales. Temperature changes in the bales were measured either with mercury-in-steel thermographs or with distance-reading bimetallic thermometers inserted into the bales. Sampling methods differed from year to year; details are given under the separate sections. Moisture content was determined from a subsample either by oven-drying, or (in 1961 only) by freeze-drying.

Microbial investigations

Examination of dust

For general microbial examination of dust, 20–30 g. hay were shaken in a perforated zinc drum in a wind tunnel (Gregory & Lacey, 1963*b*). The dust removed in a wind of 4·2 m./sec. was sampled with: (1) the Cascade impactor (May, 1945) for visual examination of the wind-blown microflora (2) the Liquid Impinger (Rosebury, 1947) in 1959, and the Andersen Sampler (Andersen, 1958) in 1960 and 1961, to isolate micro-organisms in culture. Fungi were isolated on 2% malt extract agar containing 20 units penicillin/ml. and 40 units streptomycin/ml. Actinomycetes and bacteria were isolated on half-strength Oxoid nutrient agar containing 0·5 mg. actidione/ml. Fungi were incubated at 25° and 40°, and actinomycetes and bacteria at 25°, 40° and 60° (see Gregory & Lacey, 1962).

Examination of washings

In 1961 the bacteria and other organisms present were also studied by plating-out washings. On each sampling occasion one subsample was used for analysis of the wind-blown microflora as described above. Hay from a second subsample was cut into 2–3 cm. lengths, thoroughly mixed, and 10 g. shaken mechanically with sterile water for 10 min. to prepare a suspension for bacteriological analysis. Serial dilutions were plated with nutrient agar, and replicate plates incubated at 25°, 40° and 60°. Some dilutions were also plated on the acetate agar of Keddie (1951) to detect and enumerate lactobacilli. Double-layer plates were used and the cultures incubated at 35°. Anaerobes were determined by the method of Ingram & Barnes (1956) with Oxoid R.C.M. agar at 35°. Some of the cut hay was used for chemical analysis and moisture determinations as described below.

Biochemical investigations

In 1960, 30 g. of each hay sample was shredded, freeze-dried, milled and stored at 4° until analysed; in 1961 50 g. of each sample was freeze-dried and stored at −20°.

Lipids were extracted with boiling chloroform+methanol (2+1, by vol.) (1 g. dry hay/100 ml.) for 7 hr., the extracts purified by constituting in chloroform + methanol + water (80 + 20 + 2·5, by vol.) and passage through a column of Whatman cellulose powder, with 20 g. powder/g. lipid (Dr G. A. Garton, personal communication). Iodine values were determined by the method of Trappe (1938) on 1 ml. quantities of lipid extract after evaporation *in vacuo*. Lipids were analysed only in 1960.

Water-soluble extracts were made by heating 0·3 g. dry hay with 22·5 ml. water for 30 min. (Waite & Gorrod, 1959) at 90° and used to estimate glucose, soluble-N and volatile-N. Soluble-N and volatile-N were also estimated after extraction of hay with 0·1 N-HCl at room temperature for 24 hr. (Brady, 1960). Dry matter of aqueous extracts was determined by drying at 90° for 48 hr. (1960 only). In 1961, reducing and non-reducing sugars, amide-N and titratable acidity were also estimated in the aqueous extracts.

Glucose was specifically estimated by glucose oxidase (Huggett & Nixon, 1957) after removing the interfering colour with 5% (w/v) $ZnSO_4$ + 0·3 N-NaOH. The preparation of crude glucose oxidase used (Sigma Chemical Co.) was free from invertase, a contaminant of some other preparations (Howard, 1959). Reducing sugars were estimated by the colorimetric method of Nelson (1944) as modified by Somogyi (1952); non-reducing sugars were hydrolysed with 1% (w/v) oxalic acid for 1 hr. at 100° and estimated by the increment in reducing sugar (Macpherson, Wylam & Ramstad, 1957).

Nitrogen was determined by a micro-Kjeldahl method; volatile-N was measured after steam distillation of 5–10 ml. extract in a Markham still in the presence of borate buffer (pH 9·5; Macpherson, 1952) amide-N was estimated by the increase in volatile-N after heating for 3 hr. at 100° in N-H_2SO_4 (Macpherson, 1952).

pH values were measured electrometrically on filtered extracts made by macerating 0·5 g. dried sample/50 ml. water in a top-drive high-speed macerator for 2 min. (in 1960). In 1961 pH measurements were made on aqueous suspensions of fresh hay. Titratable acidity was measured by titration with 0·01 N-NaOH, with phenolphthalein as indicator.

RESULTS

Experimental hay from New Zealand Field, June 1959

Batch E was baled at 42% water content on 8 June; Batch N at 28% water content on 10 June; Batch L at 16% water content on 15 June.

A mixed grass and herb ley was cut for hay on 4 June 1959 and batches baled at intervals as indicated above. Each batch of 10 bales was reasonably uniform in water content. One bale of each batch was stored under cover and sampled periodically; the remaining 9 bales were stacked in a barn and used to record temperature changes. On 10 July some of the Batch N bales in the barn were wetted by rain and 4 days later had heated to 70°. One of these bales (R) was stored under cover and sampled. A little loose hay from Batch N was piled on the

ground in the open for 4 months (Gn). Also, samples of Batches E and N were incubated in small sealed tins at 25° or 40° for 1 week.

Batch E heated to 65° in 3 days, remained steady for several days and cooled to 24° by the 14th day. The single bale used for sampling behaved similarly. Batch N heated to 40° and remained at this temperature for 2 weeks. Batch L reached 30° and remained steady during this same 2-week period; afterwards Batches N and L both cooled slowly.

Microbial changes. In Batch E the number of fungus spores increased rapidly and reached a maximum of nearly 10 million/g. dry wt. hay 7 days after baling. This number remained fairly constant over 2 months, but the species composition changed. At first, mainly thermophilic moulds, such as *Absidia* spp., *Mucor pusillus*, *Aspergillus fumigatus* and *A. nidulans*, developed. These were followed by *Humicola lanuginosa* and *H. stellata* (Bunce, 1961) and later still by *Scopulariopsis brevicaulis*. In Batch N, the number of fungus spores reached a higher maximum than in Batch E (nearly 100 million/g. 37 days after baling), but increased less rapidly and were mainly *Aspergillus glaucus*, a mesophilic mould. Batch L was dry and had few fungus spores throughout (< 1 million spores/g.). Fungus species found in the 1959 hays are listed in Tables 1 and 2.

The small samples incubated in tins developed similarly to bales of the same water content and temperature. Batch N incubated at 25° produced many spores of *Aspergillus glaucus*; Batch E at 40° produced many thermophilic fungi. Specimen Gn developed many spores of Cladosporium, *Tricothecium roseum*, Penicillium and other mesophilic moulds.

Both Batch E and bale R showed a rapid and striking increase in actinomycetes and bacteria associated with self-heating of the hay to about 60° (nearly 100 million organisms/g. after 7 days). By contrast Batches N and L developed few actinomycetes (rarely exceeding 1 million/g.). Many mesophilic actinomycetes and bacteria developed in Gn.

Experimental hay from West Barnfield, July 1959

An Italian rye-grass and red clover ley was cut on 7 July 1959 and 6 bales were made on 10 July with 25% water content (D); the remaining grass was wetted by rain in the field. On the morning of 13 July some of the wettest was placed in a Polythene bag at 64% water content (T1) and stored in the laboratory. The rest was baled later on the same day at 26% water content (T2). D reached a maximum of 40° 7 days after baling and maintained this for 4 days. T1 reached 30° for several days and T2 reached 45° in 7 days and then gradually cooled.

Microbial changes. D developed a similar fungus flora to N of the 'New Zealand' field series; with a maximum of 40 million spores/g., mostly *Aspergillus glaucus*. T1 had a maximum of 12 million spores/g., mostly Cladosporium and *Tricothecium roseum*. T2 produced a maximum of 48 million spores/g., mainly *A. glaucus* with many Cladosporium.

An interesting result from this series, and also from Gn of the previous series is the development of *Tricothecium roseum*, Cladosporium, Epicoccum, Alternaria and *Acremoniella atra* in hay that got wet and then took a long time to dry in the field. Few actinomycetes and bacteria developed in D and T2 but T1 had many mesophilic, with a few thermophilic actinomycetes.

Table 1. *Presence of spores in experimental hays counted on cascade impactor slides*

Details for 1961; summaries for 1959, 1960 and 1961. Brackets ( ) indicate number of samples containing 2 million spores or more/g. dry wt. hay.

	Details of 1961 hays								Summaries		
	A	B	SA	SA 25	SB	F	G	Lu	1961 Batches	1960 Batches	1959 Batches
	No. samples tested								No. batches tested		
	26	13	22	8	11	24	8	5	8	8	10
	No. samples containing spores								No. batches containing spores		
Actinomycetes	25 (7)	9	20 (2)	8 (5)	7	24 (16)	8 (1)	5 (2)	8 (6)	8 (7)	10 (8)
Acremoniella	0	0	0	0	0	0	0	0	0	2	3
Alternaria	0	0	0	0	0	0	0	0	0	0	4
Aspergillus and *Penicillium* type	15 (8)	2	7	8 (4)	1	20 (8)	4	4 (2)	8 (4)	8 (7)	10 (5)
Aspergillus glaucus	0	0	0	0	2 (1)	0	5 (4)	0	2 (2)	1 (1)	6 (4)
Botrytis spp.	0	0	0	0	0	0	0	0	0	2 (1)	0
Chaetomium sp.	0	0	0	0	1	0	0	0	1	2	1
Cladosporium sp.	8	5	6	1	6	11	4	3	8	8	9 (3)
Epicoccum	0	0	0	0	0	0	0	0	0	2	2
Fusarium	0	0	0	0	0	0	0	0	0	0	1
Helminthosporium sp.	0	0	0	0	0	1	0	0	1	0	1
Humicola lanuginosa	8 (2)	0	10 (2)	6 (2)	0	16 (4)	0	1	5 (4)	7 (4)	4
H. stellata	0	0	0	1	0	0	0	1	2	2 (1)	4 (1)
Mucoraceae	21 (10)	2	6	7 (2)	1	21 (12)	6 (1)	3 (1)	8 (5)	7 (3)	8 (2)
Scopulariopsis brevicaulis	0	0	0	1	0	0	0	0	1	2 (1)	3 (1)
Sporotrichum sp.	0	0	0	0	0	0	0	0	0	0	1
Trichothecium roseum	0	0	1	1	0	1	0	0	3	5	4 (1)
Myxomycetes (?)	1	0	0	0	0	4	1	0	3	1 (1)	6
No. of categories	6 (4)	4	6 (2)	8 (4)	6 (1)	8 (4)	6 (3)	6 (3)	12 (5)	13 (8)	17 (8)

Table 2. *Isolation of micro-organisms from experimental hays using the Andersen sampler*

Details for 1961; summaries for 1959, 1960 and 1961. Brackets ( ) indicate number of samples with over 25 isolations, * = isolated at 25° and 40°; † = isolated at 40° only; ‡ = included with 40° whitish actinomycetes.

	Details of 1961 hays								Summaries		
	A	B	SA	SA25	SB	F	G	Lu	1961	1960	1959
	No. samples								No. batches		
	26	13	22	8	11	24	8	5	8	8	10
Fungi											
Absidia spp.*	20 (15)	9	17 (1)	9 (2)	7	23 (10)	8 (1)	4 (3)	8 (6)	8 (6)	10 (2)
Acremoniella atra	0	0	0	0	0	0	0	0	0	1	1
Alternaria sp.	2	0	0	0	1	0	0	1	3	4	1
Aspergillus flavus	3 (1)	2	1	2	0	1	0	1	6 (1)	0	0
*A. fumigatus**	22 (7)	9 (1)	17 (1)	8 (3)	9	24 (7)	7 (1)	5 (2)	8 (7)	8 (6)	9 (2)
A. glaucus	19 (13)	9	16 (5)	7 (4)	9 (1)	23 (12)	8 (5)	5 (4)	8 (7)	8 (5)	5 (4)
*A. nidulans**	16 (4)	5	7 (1)	6 (1)	3	20 (7)	3 (1)	4 (3)	8 (6)	5 (3)	8 (1)
A. niger	3	0	0	2	0	2	1 (1)	0	4 (1)	1	2
*A. terreus**	0	0	0	5 (1)	0	0	0	1	2 (1)	2	1
A. versicolor	0	0	0	5 (1)	0	0	0	1	2 (1)	5	2
Botrytis sp.	1	6	4	5 (1)	5	6	4	0	7 (1)	8	2
Candida sp.	2	8	3	1	6	5	8	2	8	7	6
Cephalosporium sp.	0	1	0	0	0	0	0	0	1	1	5
Chaetomium sp.	1	2	2	2	3	4	0	1	7	3	0
Cladosporium sp.	24 (2)	12 (4)	22 (4)	5	10 (3)	22 (5)	7 (3)	5 (4)	8 (7)	8 (2)	10 (2)
Epicoccum sp.	0	0	0	0	0	0	0	1	1	5	3
Fusarium sp.	0	0	0	1	0	0	0	0	1	1	2
Geomyces (type)	0	1	0	0	0	0	0	0	1	2	0
Hemispora stellata	0	0	1	1 (1)	1	1	1 (1)	0	5	2	0
Humicola lanuginosa†	6 (1)	3	15 (6)	7 (3)	2	12 (5)	1	2	8 (4)	7 (1)	1
H. stellata	0	0	0	0	0	0	0	0	0	0	1 (1)
*Mucor pusillus**	20 (15)	7	12	7 (1)	5	22 (14)	6 (1)	5 (3)	8 (5)	7 (3)	5 (2)
M. spinosus	0	0	0	0	0	0	0	0	0	0	1
Paecilomyces sp.*	14 (7)	5	8 (1)	7 (1)	3	21 (14)	3	5 (2)	8 (5)	6 (3)	3 (1)
Papularia sp.	0	0	0	0	1	0	0	0	1	4	1
Penicillium spp.	13 (2)	11 (1)	11	5 (1)	7	13 (1)	2	3 (1)	8 (5)	8 (3)	10 (6)
Pullularia (*Aureobasidium*) sp.	0	0	0	0	0	0	0	2	1	2	0

Table 2 (*cont.*)

	Details of 1961 hays								Summaries		
	A	B	SA	SA 25	SB	F	G	Lu	1961	1960	1959
	No. samples								No. batches		
	26	13	22	8	11	24	8	5	8	8	10
Fungi (*cont.*)											
Rhizopus sp.	3	0	2	0	0	0	1	1	4	1	2
*Scopulariopsis brevicaulis**	0	0	1	2 (1)	0	0	0	0	2	5 (1)	5 (2)
Sporendonema	0	0	0	0	0	0	0	0	0	2	0
Sporotrichum	0	0	0	0	0	0	0	0	0	3 (1)	0
Stysanus	0	0	0	0	0	0	0	0	0	3	0
Trichoderma viride	1	0	0	0	1	0	0	0	2	2	2
Trichothecium roseum	0	0	0	2	0	1	0	0	2	5 (1)	4 (2)
Yeasts	10	9	12	1 (1)	9	7 (1)	5	1 (1)	8 (3)	5	4 (1)
Verticillium lateritium	0	0	0	0	0	0	0	0	0	0	1
Sterile mycelium and unidentified	14	4	15	5	5	10	6	2	8	7	7
No. of categories	19 (10)	16 (3)	18 (7)	22 (14)	18 (2)	18 (10)	16 (8)	20 (9)	30 (15)	34 (12)	29 (12)
Bacteria											
25°: orange	12 (1)	0	2	7 (4)	2	18 (11)	2 (1)	5 (4)	7 (5)	8 (4)	9 (5)
others	26 (1)	12	22 (2)	7 (2)	11	22 (6)	8 (2)	5 (5)	8 (6)	8 (5)	8 (7)
40°: *Bacillus licheniformis*	12	8	17 (2)	7 (3)	5	18 (3)	5	5	8 (2)	8 (2)	7 (2)
others	21 (1)	11	19	8 (3)	10	21 (9)	8	5 (3)	8 (4)	8 (3)	9 (6)
No. of categories	4 (3)	4	4 (2)	4 (4)	4	4 (4)	4 (2)	4 (3)	4 (4)	4 (4)	4 (4)
Actinomycetes											
25°: incl. *Streptomyces griseus*	23 (4)	10	18	8 (1)	6	21 (5)	5 (1)	5 (1)	8 (5)	8 (5)	10 (5)
40°: whitish incl. *S. fradiae. Micromonospora vulgaris*	26 (5)	12 (1)	22 (3)	8 (6)	10	24 (5)	8	5 (2)	8 (6)	8 (7)	10 (8)
blue incl. *Thermopolyspora glauca*	4	4	3	7 (5)	3 (1)	3	1	3	8 (2)	7 (2)	1 (1)
grey incl. *S. griseo-flavus*, *S. olivaceus*, *S. thermoviolaceus*	19 (1)	12	19	1	11	15	5	4	8 (1)	8 (2)	‡
thin	5 (3)	1	2 (1)	5 (1)	1	4 (2)	0	1	7 (4)	0	0
small incl. *T. polyspora*	12 (4)	6	13 (5)	7 (6)	4 (1)	15 (9)	3	3 (3)	8 (6)	8 (7)	0
60°: white incl. *M. vulgaris*	25 (4)	13	22 (13)	8 (8)	11	24 (8)	8	4 (1)	8 (5)	8 (8)	0
small incl. *T. polyspora*	8 (3)	0	6 (3)	0	0	14 (7)	1	0	4 (3)	7 (5)	0
No. of categories	8 (7)	7 (1)	8 (5)	7 (6)	7 (2)	8 (6)	7 (1)	7 (4)	8 (8)	7 (7)	3 (3)
Total no. of categories	31 (20)	27 (4)	30 (14)	33 (24)	29 (4)	30 (20)	27 (11)	31 (16)	42 (27)	45 (23)	36 (19)

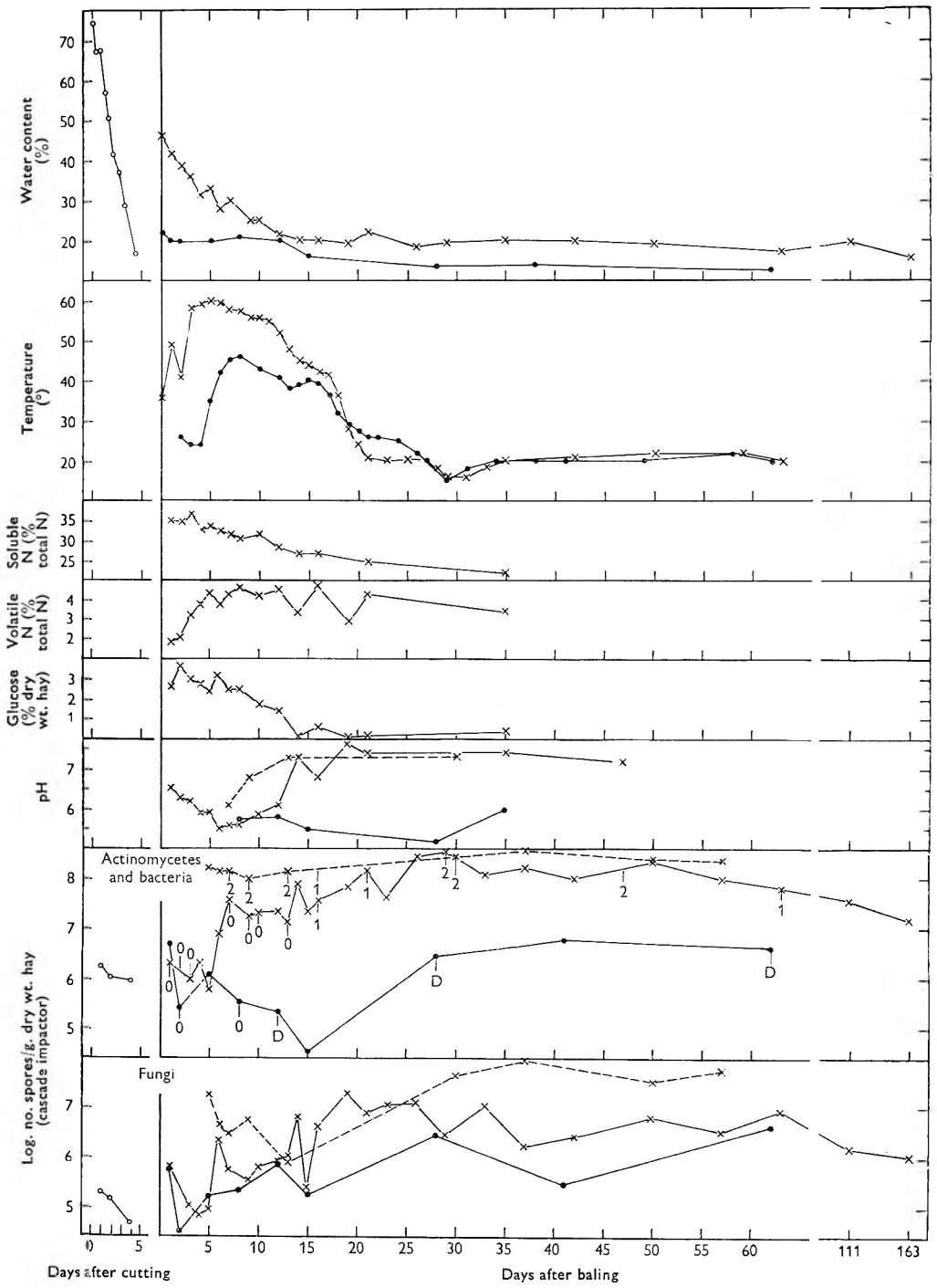

Fig. 1*a*. Changes in wet and dry baledh ays of Great Field made in June 1960. ×——×, Batch W; ×– – –×, Batch W2; ●——●, Batch Y; ○——○, grass drying in the field. Serological reactions are shown on the graph for actinomycetes and bacteria: the figure indicates the number of precipitin bands in gel-diffusion tests; the letter D indicates a diffuse precipitin band.

Experimental hay from Great Field, June 1960

Batch W baled at 46 % water content on 1 June; Batch W2 (fast moulding part of top bale of W); Batch X baled at 39 % water content on 1 June; Batch Y baled at 25 % water content on 2 June; Batch V baled at 26–38 % water content on 3 June.

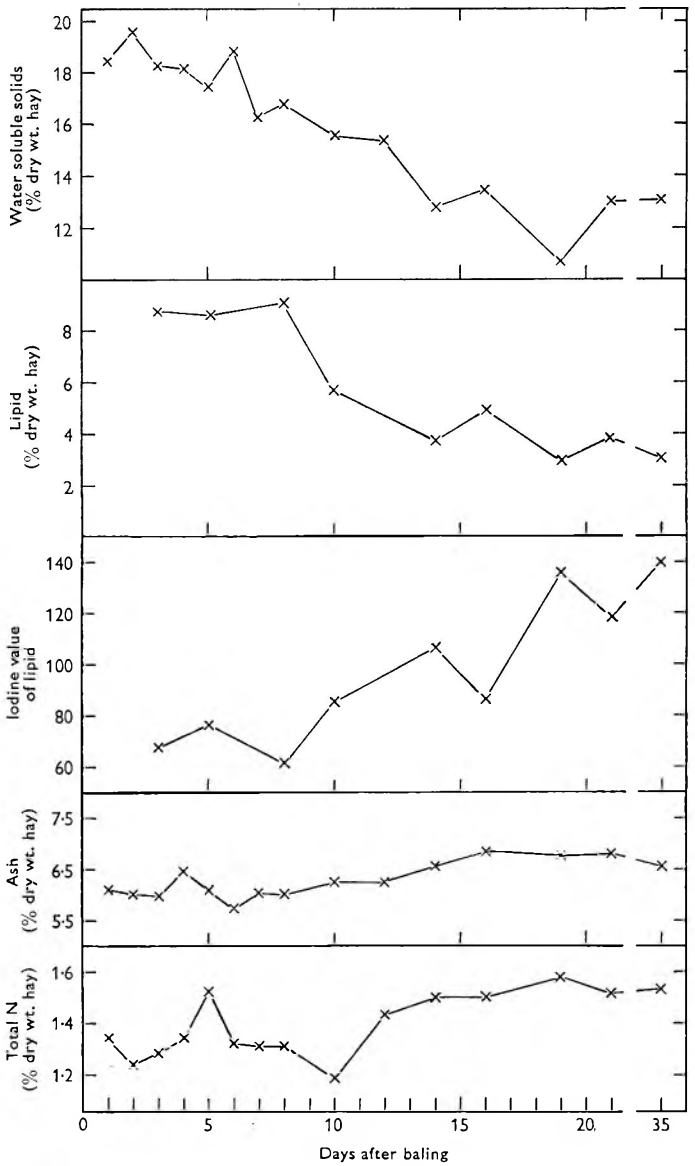

Fig. 1*b*. Chemical changes in W hay of Great Field, made in June 1960.

The timothy and fescue grass ley in Great Field was cut for hay on 30 May and baled at intervals as shown above. The drying and damping of this grass by dew was studied by Long (1961). Each batch of experimental hay, which consisted of 7 bales, was fairly uniform except for V (26–38 % water content), and to a lesser

extent X (39 % water content). Batches of bales were stored in a barn in separate stacks with the bales arranged in layers of 3, 3 and 1. Temperature records and samples were taken from the centre bale, the single bale on top being rolled aside for sampling, and each day's sample of about 30 g. was taken next to the previous sampling position. During the early sampling of W, the side of the top bale was seen to be moulding quickly, so extra samples were taken from this area (W2).

Temperature changes. The temperature of the damp hays rose rapidly to maxima of: V = 56°, W = 60°, X = 59° and Y = 46°.

Microbial changes. Fig. 1*a* shows changes in number of spores after baling W, W2 and Y. Batch X and the damp bales of V behaved like W, and the drier bales of V like Y.

Fungi. Fungus spores increased rapidly in W and W2, consisting mainly of thermophilic moulds (Tables 1, 2) and including *Aspergillus* spp., *Absidia* spp., *Mucor pusillus*, *Humicola lanuginosa*, *H. stellata*, and then *Paecilomyces variotii*. Batch X and the damp bales of V moulded slightly slower and produced fewer spores (maximum of 27 million/g. in X; 10 million in V). Batch Y was a less good control for the series than L had been in 1959, because it moulded with *Aspergillus glaucus* and a few thermophilic moulds.

Actinomycetes and bacteria. The increase in actinomycetes and bacteria in W and W2 is associated with the high temperature reached during self-heating (Fig. 1*a*). Y heated less and had few spores. In X the most counted was 134 million/g. dry wt., and in the damp bales of V 208 million. Table 2 gives more details of the actinomycetes isolated (and see Discussion).

Biochemical changes (*Batch W*). During the first 6 days after baling, while the temperature rose to and was maintained near 60°, the pH value decreased from 6·5 to 5·5, and volatile-N increased rapidly, corresponding to the increase in actinomycetes and bacteria (Fig. 1*a*). Browne (1929) suggested that micro-organisms produce unsaturated compounds during self-heating of hay, but in W the iodine number of the lipids changed only slightly while the temperature was at its highest; an appreciable rise occurred only after 14 days when the temperature had dropped below 50° and numbers of fungi had increased considerably (Fig. 1*b*). Increase in fungi was also associated with losses in glucose, lipid, soluble dry matter and soluble-N, and with a rise in pH value, which rose to 7·6; but there was no corresponding increase in volatile-N, which suggests metabolism of fatty acids by fungi rather than increased ammonia production. A slight increase in ash content occurred during moulding, associated with the loss in glucose; the slight increase in total-N accompanying the increase in ash content was probably not a true increase either, but also reflected the loss of dry matter as glucose.

Experimental hay from Great Field, September 1960

Batch S heaped under cover at 65 % water content, on 10 September; Batch P baled at 17 % water content on 14 September and stored in barn; Batch Q baled at 17 % water content on 14 September, left out in rain until 13 October.

The timothy and fescue ley in Great Field was cut for a second hay crop on 8 September 1960, crushed by forage harvester to aid drying, and tedded. A pile of grass (about 65 % water content; S) about 1 m. diameter and 1 m. high was made on 10 September. This was covered with a metal sheet, and a thermograph inserted.

The temperature rose to 52° in 4 days, and cooled to 18° by the 10th day after piling. Traces of rain fell on 11 and 12 September, and the rest of the crop in the field had already moulded a little when baled on 14 September. To test the effect of rain wetting bales left out in the field, 4 bales (Q) were left in the open, and by the time they were moved to the barn they had received 5 in. (127 mm.) of rain. The bale temperature usually fell during rain but rose again soon after the rain ceased, reaching maximum of 40° on 23 September in the open, and on 15 October in the barn, on both occasions a short time after a thorough soaking. Four bales taken to the barn directly after baling (P) reached a maximum of 22°.

Microbial changes

Fungi. Specimens S and Q developed the usual thermophilic fungi. On the 10th day after piling S had 20 million spores/g., 11 million of which were *Humicola lanuginosa*. Q contained 21 million spores/g., including those of a pink Sporotrichum-like form growing at 40° but not at 25°. *Trichothecium roseum* was isolated from 4 of the 6 samples from Q. Few fungi developed in P.

Actinomycetes and bacteria. The striking rise in temperature of the pile S was associated with a rapid increase in actinomycetes to 89 million/g. by the 10th day after piling. Of the 25 million spores/g. in Q, many were not thermophilic. P developed few actinomycetes.

Experimental hay from Great Field, June 1961

Batch A baled at 30 % water content on 5 June, stored in barn; Batch SA stacked at 30 % water content on 5 June, stored under sheet in field; Batch B baled at 15 % water content on 6 June, stored in barn; Batch SB stacked at 15 % water content on 6 June, stored in barn.

The timothy and fescue grass ley in Great Field (used in 1960) was again cut for hay on 31 May 1961. The grass was very thick in the stand and although it was crushed twice and tedded several times it dried slowly and unevenly. These changes in farm practice made the batches of hay in 1961 more heterogeneous than in the previous years, and there was greater irregularity in samples taken for analysis. Each sample from the bales was taken by removing about five slices. From one of these, a sample was taken for blowing in the wind tunnel and the rest was mixed before sampling for bacteriological and biochemical examinations (Methods). The remaining four slices were sent to Dr J. Pepys, Institute of Diseases of the Chest, Brompton Hospital, London, for immunological work.

The wet (SA) and dry (SB) stacks were made 15 ft. diameter and 12 ft. high, and each was equipped with a horizontal 6 in. diam. asbestos pipe about 2 ft. from the ground and extending from the periphery to the centre. Samples were periodically withdrawn from the centre of a stack through the pipe by using a large hook bent for the purpose from $\frac{1}{2}$ in. square steel bar. After sampling, the outer end of the pipe was closed with a wad of hay to restrict aeration and minimize heat loss. Samples were taken from bales and stacks frequently over a period of 80 days (Figs. 2*a* to 3*b*). Finally, in early December when 186 days had elapsed, the wet stack (SA) was cut open and single samples taken from eight different parts (Fig. 4). Table 3 shows the results of the analyses on these samples.

Temperature and moisture changes. The temperature of A rose to 55° in 1 bale

but, as the hay was unevenly dried, some areas in this batch did not heat above 48° (compare V, 1960). Moisture content fell, but, as Fig. 2*a* shows, different parts of any one bale differed in moisture content (the samples from days 1–4 after baling were from the same bale). B hardly heated at all and the moisture content decreased only slightly. Temperatures in the stack SA were recorded with two dial thermometers

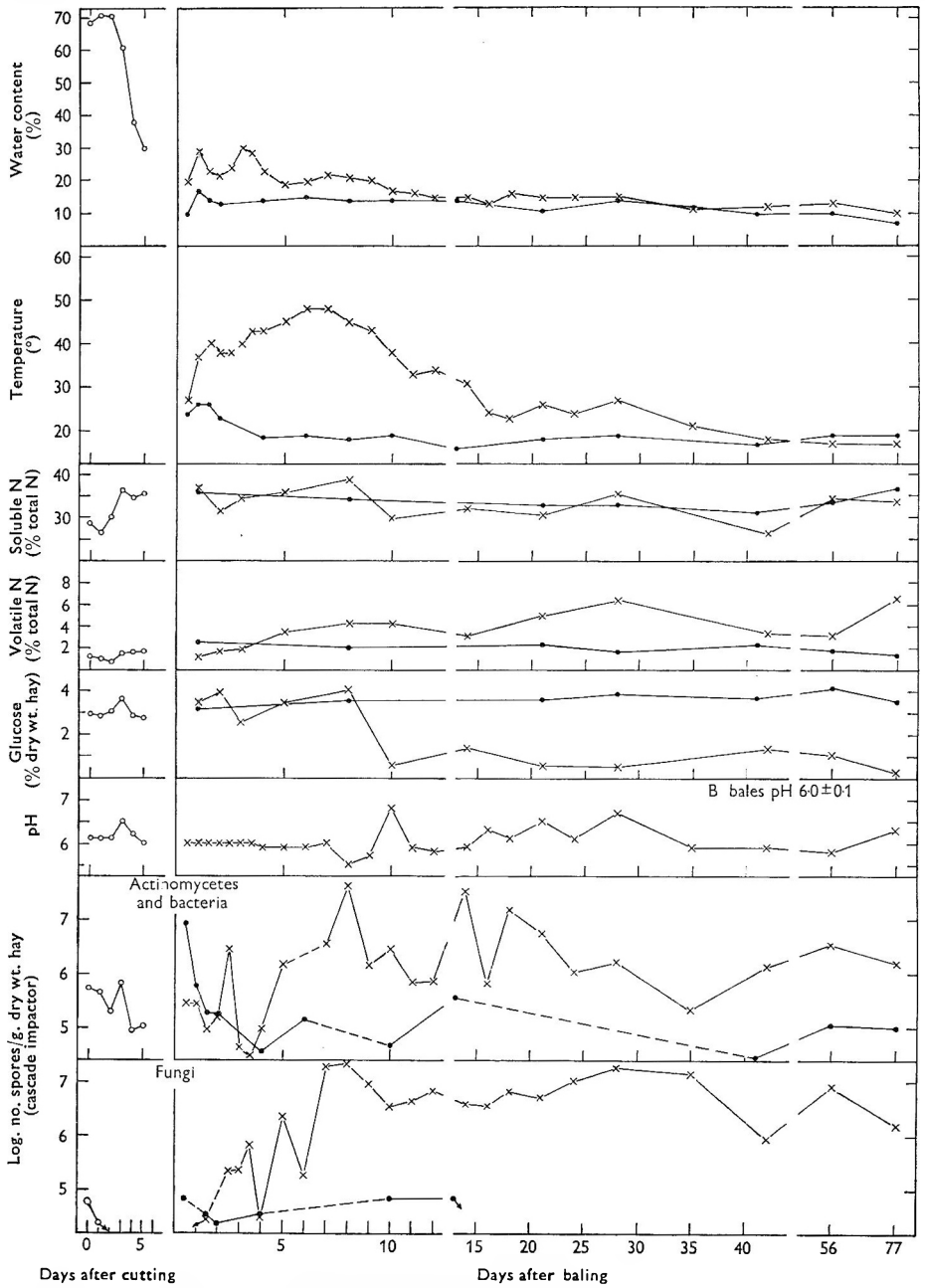

Fig. 2*a*. Changes in wet (A) and dry (B) baled hays of Great Field, made in June 1961. ×, Wet bales (A); ●, dry bales (B); ○, grass drying in the field.

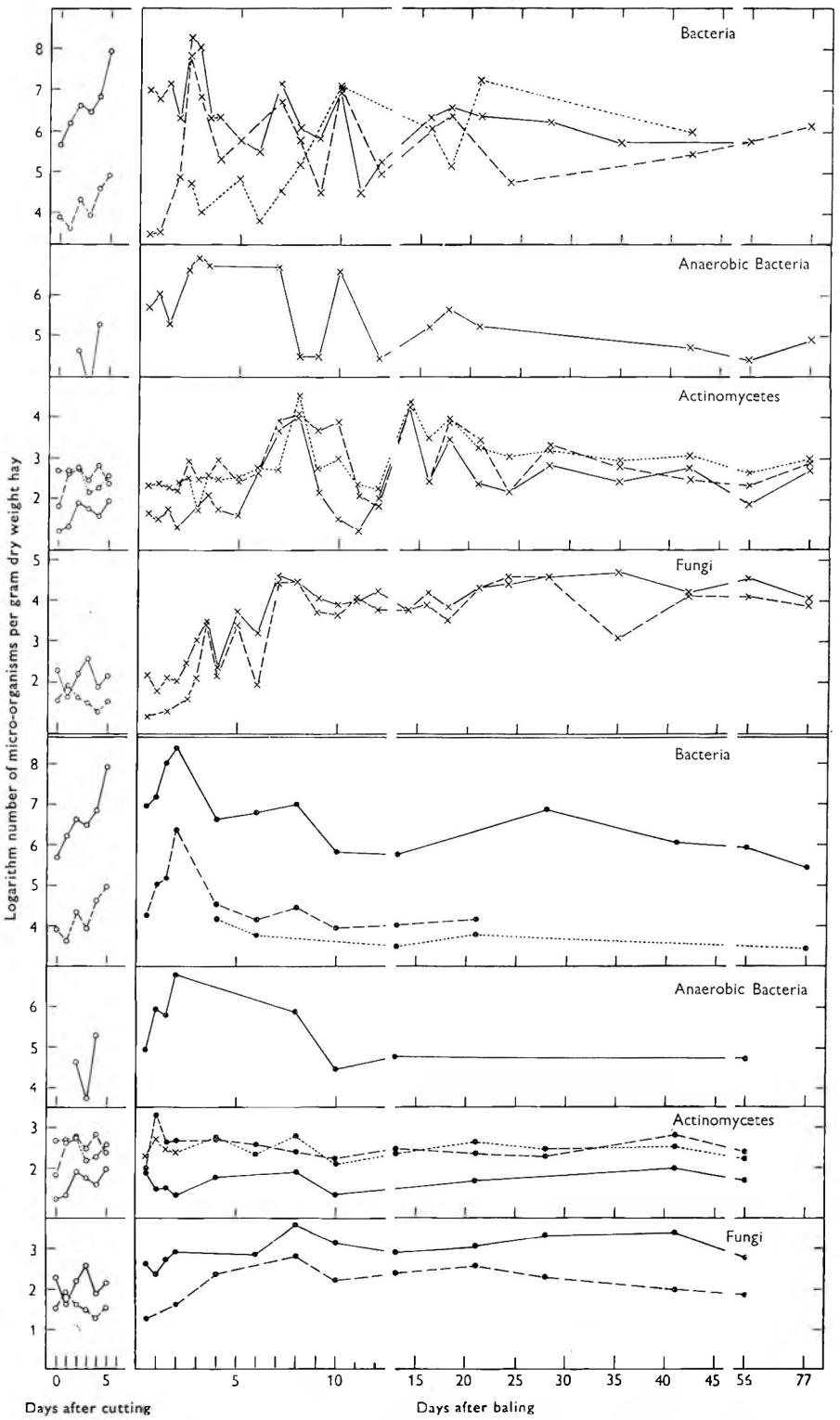

Fig. 2*b*. Changes in the microflora of wet (A) and dry (B) baled hays of Great Field, made in June, 1961. ×, Wet bales (A); ●, dry bales (B); ○, grass drying in the field. ——, 25°; – – –, 40°; . . . ., 60°.

one 5 ft. long, the other 3 ft., read daily. During self-heating of SA the temperature rose much as in the corresponding set of bales (A); on each day the higher temperature was recorded by the thermometer nearer the centre of the stack

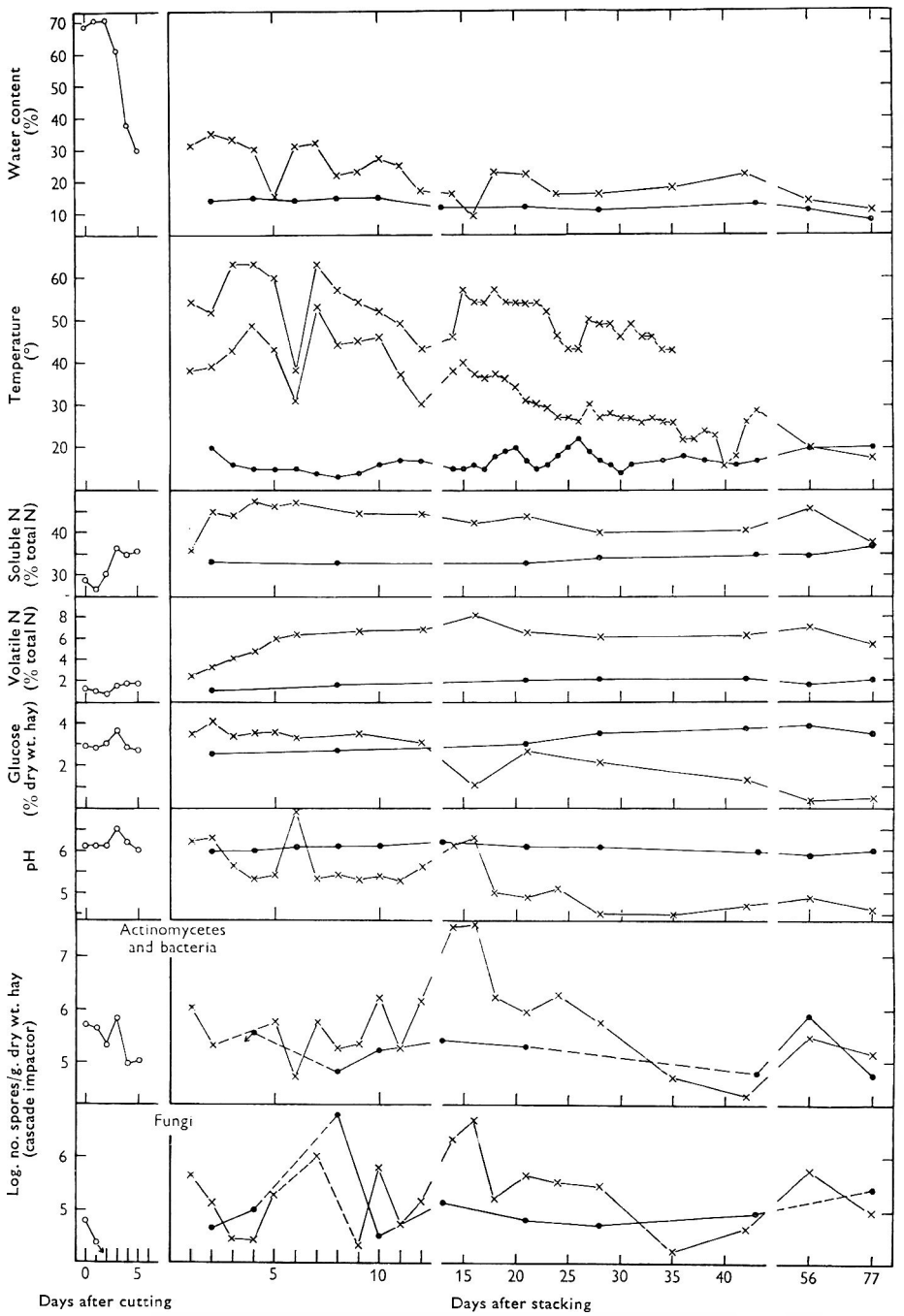

Fig. 3*a*. Changes in wet (SA) and dry (SB) stacked hays of Great Field, made in June 1961. ×, Wet stack (SA); ●, dry stack (SB); ○, grass drying in the field.

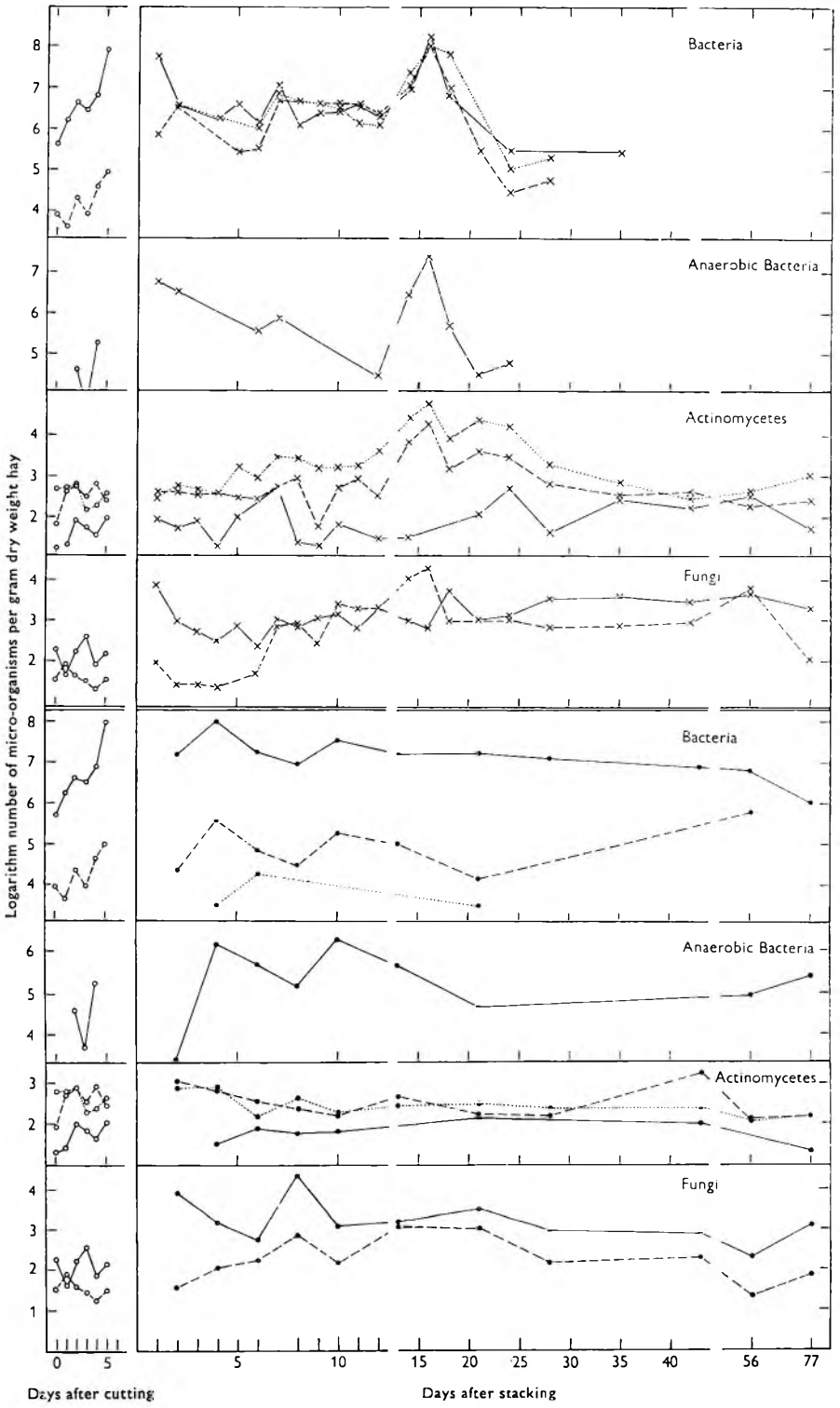

Fig. 3*b*. Changes in the microflora of wet (SA) and dry (SB) stacked hays of Great Field, made in June 1961. ×, Wet stack (SA); ●, dry stack (SB); ○, grass drying in the field. ——, 25°; – – –, 40°; . . . ., 60°.

(Fig. 3*a*). Stack SA in the open field fluctuated much more than SB in the barn with outdoor temperature and wind velocity. The moisture content fell eventually to less than 20 %, but, as in the bales, there were large variations between successive samples. The temperature of stack SB fluctuated little, and the moisture content remained more or less steady.

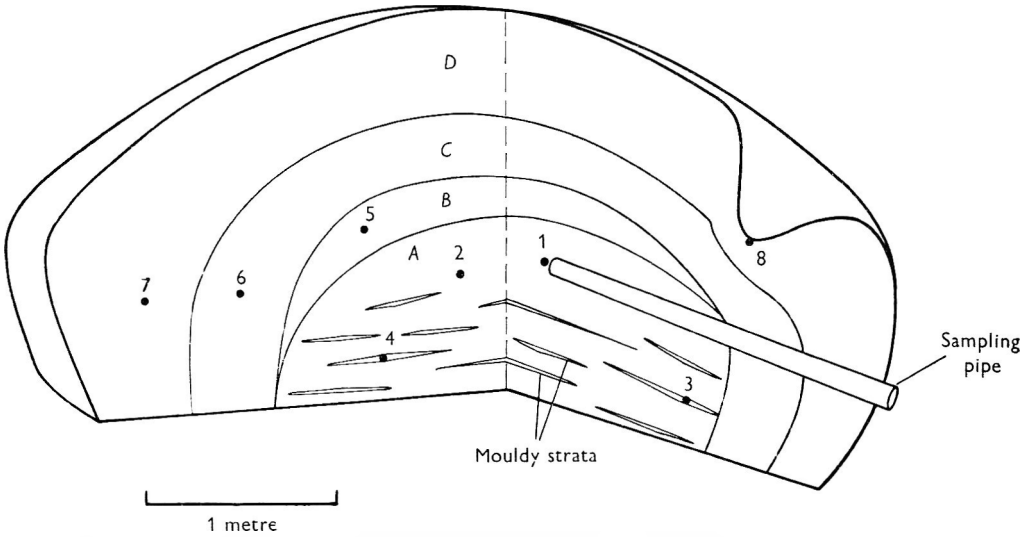

Fig. 4. Schematic diagram of Stack SA after opening. A, Brown hay with mouldy strata; B, grey-brown hay; C, greenish-brown hay; D, greenish-grey hay.

Microbial changes

Fungi. Thermophilic fungi developed in the wetter parts of the A bales. Most samples had more Absidia and Mucor type spores than Aspergillus. *Humicola lanuginosa* spores were numerous and reached 8·6 million/g. *Paecilomyces variotii* developed a little later than the other thermophilic moulds. The B bales had few fungi; Cladosporium was commonest, but *Aspergillus glaucus*, *A. fumigatus*, and *Penicillium* spp. were numerous in some samples. In SA *H. lanuginosa* was the most abundant fungus in the visual counts, but it grows slowly and is under-represented on the Andersen sampler plates, being overgrown by others, including *A. fumigatus*, Absidia and *Mucor pusillus*. The series of SA samples taken after 186 days (Fig. 4, Table 3) show the differences between parts of the stack. The areas of 'brown' hay had few fungi (SA 25/1 and 2); the white hay (SA 25/3) and a greyish-brown hay just outside the brown core (SA 25/5) had many more. SB, like B, had very few fungi.

Actinomycetes. The Cascade impactor counts of actinomycete and bacterial spores differed greatly, but in many samples of A and SA they were very large. Table 2 shows the abundance of actinomycetes isolated. A and SA had many thermophilic actinomycetes, e.g. *Micromonospora vulgaris*, whereas B and SB had only a few.

Bacteria. During the 5 days that the hay lay drying in the swath the numbers of bacteria able to grow at 25° increased steadily. This flora consisted of many different species. Few bacteria grew at 40° and none at 60° during this period. Neither

Table 3. *Characteristics of hays (Great Field, June 1961) from opened stack SA*

Sample	Moisture (%)	pH	Glucose (% dry wt.)	Soluble-N (% of total-N)*	Volatile-N (% of total-N)	Ash (% dry wt.)	Bacteria growing at 25° Millions/g. dry wt. hay	Bacteria growing at 40° Millions/g. dry wt. hay	Bacteria growing at 60° Millions/g. dry wt. hay	Cascade impactor: Aspergillus Millions/g. dry wt.	Cascade impactor: Mucor Millions/g. dry wt.	Cascade impactor: Humicola Millions/g. dry wt.	Cascade impactor: Total fungi Millions/g. dry wt.	Cascade impactor: Actinomycetes + bacteria Millions/g. dry wt.	Remarks
SA 25/1	14	4·8	0·26	39 (37)	3·5	7·3	0·29	0·03	0·03	0·04	0·04	0·04	0·12	0·86	Brown hay. From same position as sequential samples
2	14	4·8	0·68	41 (39)	6·2	7·2	3·78	0·38	3·29	0·29	0·0	0·0	0·29	0·01	Brown hay from core
3	15	7·8	0·12	37 (28)	4·5	9·6	180	146	—	2·78	2·03	15·25	20·55	281	60° organisms too numerous to count. Brown hay with white areas.
4	13	7·4	0·06	47 (35)	9·7	9·2	307	356	11400	0·14	0·04	0·66	0·95	142	Brown hay with white areas.
5	14	6·1	0·69	36 (37)	6·1	7·4	7·85	20·1	16·6	19·82	0·29	0·19	20·30	3·35	Grey-brown hay; mouldy region just outside core
6	14	5·9	2·30	38 (41)	5·5	6·7	0·29	0·12	0·0	3·55	7·49	0·03	11·07	1·30	Greenish-brown hay, less mouldy than sample 5
7	27	7·1	0·09	27 (23)	3·3	7·4	1290	7·67	0·0	8·06	1·52	0·0	11·57	2·27	Greenish-grey, with evidence of low temperature decomposition in outermost region of stack
8	16	7·5	0·09	44 (33)	8·3	9·7	26·8	1260	1450	0·38	0·58	2·55	3·51	45·4	Brown hay with white areas below point of rainwater entry. Similar to samples 3 and 4
SB/7	12	6·1	3·02	33 (35)	2·0	6·7	16·2	0·01	0·003	0·0	0·0	0·0	0·06	0·19	SB/7. Good greenish dry hay

* Figures in brackets refer to acid extracts.

anaerobes nor lactobacilli were detected before 2 days had elapsed, and later their numbers rarely exceeded 10^5/g. Low numbers of lactobacilli on herbage were reported by Stirling (1953).

In the control bales of dry hay (B series) bacteria isolated at 25° increased from $8{\cdot}89 \times 10^6$/g. to $2{\cdot}33 \times 10^8$/g. in the 2 days after baling, and decreased to $6{\cdot}4 \times 10^5$/g. during the next 8 days. The peak occurred 1 day after 25° was reached in the bales, and this flora was also very mixed in composition. Bacteria were fewer at 40° but numbers fluctuated as at 25°. A few bacteria able to grow at 60° were found in 9 samples. Anaerobes occurred in most samples in numbers intermediate between those for bacteria at 25° and 40°, and followed the same general trend. The course of events in the dry stack (SB series) was similar in all respects to that in the dry bales.

In the wet bales (A series) bacteria growing at 25° remained fairly numerous throughout the experiment and were not greatly influenced by the considerable fluctuations in bale temperature. However, in the early stages the flora was very mixed, with a tendency for organisms forming whitish-buff and yellowish colonies to predominate. Later, whitish punctiform colonies were commonly found. Bacteria at 40° were scarce at the time of baling but increased rapidly to a maximum 2 days later, when the temperature of the hay was 38°, and remained moderately numerous thereafter. During the first 6 days after baling very few organisms grew at 60°, but an increase was noted on the 7th day when the temperature of the hay was 50°. In the first few days after baling small white and buff colonies frequently grew at 40°, but 10 days after baling, and coincident with the second temperature peak, the flora took on characteristics that we now associate with growth at high temperatures: flat, thin, white, overlapping colonies with filamentous margins forming almost the sole constituents of this flora. The 60° flora was essentially similar. The bacterium forming these colonies was identified as *Bacillus licheniformis*.

During the first 7 days after baling when the numbers of all micro-organisms were rising, lactobacilli were usually detected on acetate agar but usually fewer than 10^5/g. The numbers were also rather variable, presumably because of the heterogeneity of the material. After 10 days these organisms could not be estimated because the plates of acetate agar always became covered with a dense growth of mucoraceous fungi during overnight incubation. Typically, the pH value of the medium with this fungus cover was 8·0–9·6 as indicated by drops of thymol blue solution placed on the surface of the plate. The presence of ammonia in such alkaline media was indicated by Nessler reagent. Plates covered with fungi began to appear shortly after a marked increase in fungi (at 25° and 40°) and a trend to greater pH values of the hay (10 days after baling). Keddie's acetate agar was not used in this series after the first 3 weeks of sampling.

The bacterial population of the wet stack (SA series) behaved quite differently from that of the dry stack and from that of the wet bales. Bacteria at 60° developed quickly in the centre of the stack and reached $3{\cdot}58 \times 10^6$/g. after 24 hr. A peak of $1{\cdot}11 \times 10^8$/g. was reached on the 13th day, and a subsequent decrease in numbers coincided with a gradual decrease in temperature. The numbers of bacteria at 40° and 25° followed the same course as those at 60° and were of the same order. All the bacteria at these three incubation temperatures formed the same filamentous type of colony of *Bacillus licheniformis* described above. Bacteria likely to be the

B. calfactor of Miehe (1907) were not found on the plates. Anaerobes also followed the same trend as the bacteria at 25°, 40° and 60°, but, apart from a peak of $2{\cdot}75 \times 10^7$/g. 13 days after stacking, the numbers were low. Lactobacilli were found in 8 of the sequential samples but they never exceeded $3{\cdot}62 \times 10^4$/g., the estimate for the first sample. On two occasions, plates of acetate agar were spoiled by rapid growth of mucoraceous fungi and development of alkalinity. These 2 samples (SA/12 and 13) were taken when a marked increase in number of fungi and in pH was taking place. In sampling after SA/14 there was a decrease in fungi at 40° and in pH value, and lactobacilli were again detected.

In the dry stack SB, lactobacilli were detected in the first 6 samples but never exceeded $1{\cdot}18 \times 10^4$/g. SB/7 yielded fungus-covered plates and the acetate agar was not used after SB/8. In the dry bales, lactobacilli were not detected until 1 week after baling. Fungus-covered plates occurred with samples B 1/7 and 8. The occurrence of lactobacilli in the good hays was not associated with any large chemical changes in the hay or with changes in the populations of other micro-organisms.

Biochemical changes (1961). During wilting soluble-N and ash increased, non-reducing sugar decreased and reducing sugar increased (Figs. 2*a*, 3*a*, 5*a*, *b*). Sucrose, estimated by the increase in glucose formed after acid hydrolysis, decreased more rapidly than fructosan, as shown in the topmost graph of Fig. 5*a*.

After baling, soluble-N did not change much, but on stacking there was a further increase for 2 days. Volatile-N increased soon after baling and also after stacking, where a greater value was reached. Results for amide-N and total-N are not shown because the changes were small; amide-N was usually 2–3 % of total-N; the latter was similar to that of W (1960).

The pH value decreased after baling, as in 1960 (W), but less obviously because the samples were more uneven. The subsequent large pH values of 1960 were not obtained. The pH values of the stack samples decreased and remained small except where moulding had occurred, as for the sample 16 days after stacking. Mould development may be caused by access of air, as the sample 6 days after stacking was at pH 6·9: the sampling tool had jammed in the pipe on the previous day and efforts to release it led to access of air and a fall in temperature. The results for titratable acidity (Fig. 5*b*) show a clear inverse relationship to pH value, for both stack and bales.

The decrease in non-reducing sugar and increase in reducing sugar, which started during the wilting period, was maintained for a few days after baling and stacking, until the non-reducing sugar had decreased considerably. Samples from the stack with pH values less than 5·5 had comparatively high glucose and total reducing sugar content even 28 days after stacking. Where mould had developed, as for the sample 16 days after stacking, the pH value was 6·3 and both glucose and total reducing sugar were considerably diminished.

There was no substantial change in ash content after baling or stacking. The complete results are shown along with those for reducing and non-reducing sugar, because oxidative changes in sugars are most likely to be reflected in the ash content; during anaerobic breakdown to lactic acid there is no loss of dry matter, but, if there is oxidation involving loss of dry matter as CO_2, the ash content would rise correspondingly. The control bales (B) and stack (SB) showed very little change during the 90-day period of the experiment.

Correlations between biochemical and microbial changes. Statistical correlations were determined for all the common biochemical and microbial measurements on batches A and SA. In the subsequent discussion the 5% level ($P \leqslant 0{\cdot}05$) at least

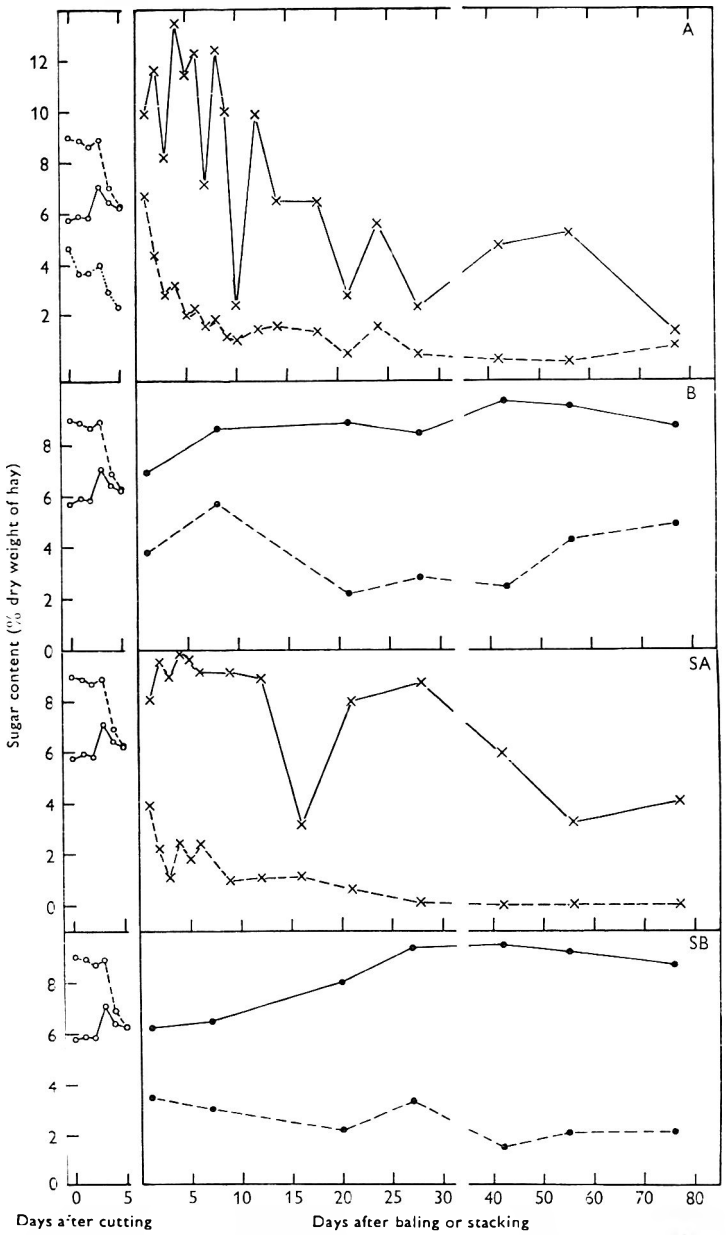

Fig. 5*a*. Reducing and non-reducing sugars of 1961 Great Field hays. ×, Wet bales (A) or stack (SA); ●, dry bales (B) or stack (SB); ○, grass drying in the field. ——, Reducing sugar; – – –, non-reducing sugar; . . . ., sucrose (shown in panel A only).

is required for a significant correlation. Volatile-N is correlated with total numbers of fungi, and 60° bacteria in the bales; also with 40° fungi and 60° actinomycetes in the stack; as noted in 1960, the 60° organisms influence the volatile-N soon after

baling or stacking. The soluble- and total-N do not correlate with any of the groups of micro-organisms. Reducing sugar, non-reducing sugar and glucose in bales are all negatively correlated with the number of 60° bacteria, and with 25° and 40°

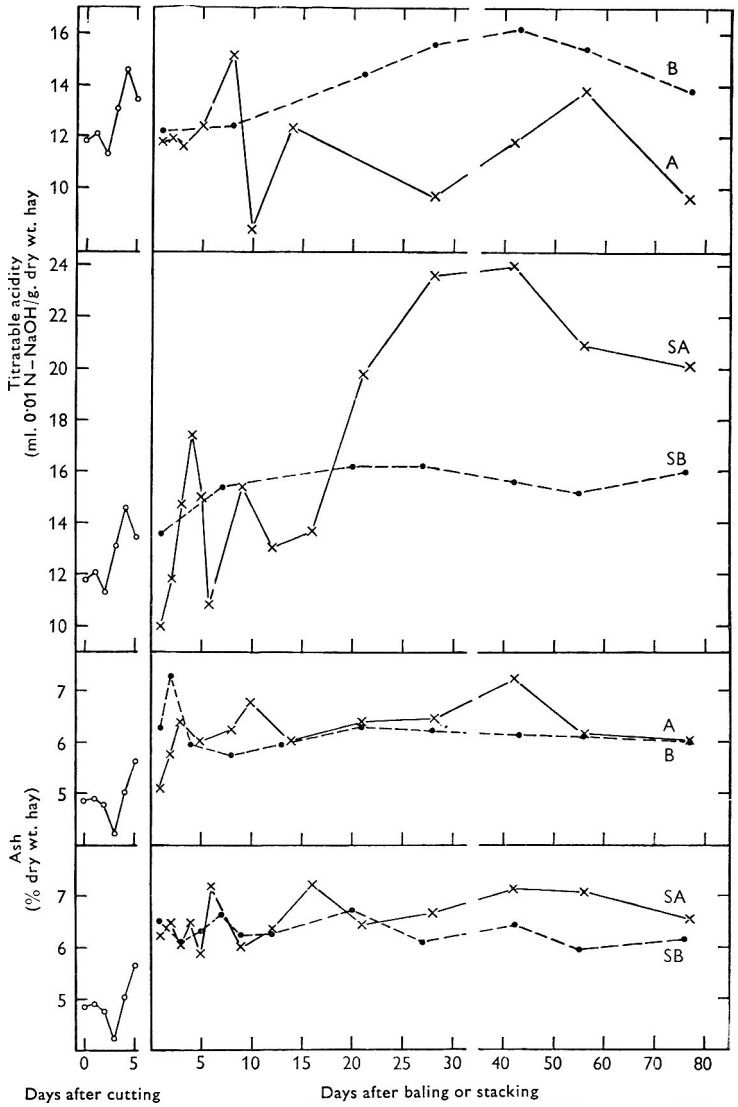

Fig. 5*b*. Titratable acidity and ash content of 1961 Great Field hays. ×, Wet bales (A) or stack (SA); ●, dry bales (B) or stack (SB); ○, grass drying in the field.

fungi; the non-reducing sugars are more readily attacked by the micro-organisms, for in addition they are negatively correlated with 40° bacteria. In the stack, 40° fungi are responsible for decrease in sugar content. Ash content is positively correlated with 40° and 60° bacteria, and with 25° fungi in the bales, and with 40° fungi in the stack; the same organisms are responsible for the changes in sugar content, suggesting that loss of dry matter, where it occurs, is mainly a loss of sugar.

Samples from stack (SA) in December 1961 (*Fig.* 4; *Table* 3). When cut open in

December 1961, stack SA consisted of several zones of different-coloured hay. A central brown core extended to about 1 m. above ground level with a diameter of 1–2 m. at its widest and lowest part. This core was surrounded by a mantle of mouldy hay, about 0·5 m. thick, which was brownish on the inner side and greenish on the outer. The outermost layer of the stack consisted of apparently good greenish hay, though this had undergone some decomposition, mainly bacterial, at low temperature (Table 3, sample SA 25/7). The structure of this stack was essentially the same as that of the damp stacks described by Truninger (1929) and Miche (1930), but the zones were less clearly defined. Some small parts of this stack were composed of hay that did not fit into any of the categories described by Truninger. Such hay occurred in thin strata running at different levels through the central core: it was brown, indicating that it had reached a high temperature, but it was also covered with a heavy deposit of fungus and actinomycete spores. This hay was, moreover, strongly alkaline and somewhat resembled the hay of the very mouldy bales. The reason for the development of mould to this extent in these regions is not clear, but it may have resulted from the uneven distribution of moisture which was characteristic of all the 1961 hay batches. At one point (sample 8) moulding followed penetration of rain into the stack after all sequential sampling had finished.

Experimental hay from Great Knott I Field, June 1961

Batch F baled at 35% water content on 20 June; Batch G baled at 20% water content on 21 June.

A mixed grass ley on Great Knott I Field was cut for hay on 19 June and baled as shown above. Unfortunately Batch F was unevenly dried and did not mould uniformly. Batches F and G bales behaved like the Batches W and Y from Great Field, June 1960. Details of these 2 batches will be published later with immunological work in connexion with Farmer's Lung disease.

Experimental hay from Lucerne plots on Foster's Field, July 1961

Batch Lu was baled at 44% water content on 19 July. A small area of lucerne on Foster's Field was cut on 19 July and placed in the barn at 44% water content, in a pile 1 m. diameter and 1·3 m. high. This heated spontaneously to 48° by the 4th day, and the temperature then gradually fell. The water content also fell, reaching 20% by the 12th day. Although almost entirely leguminous, this hay moulded in a similar manner to batch S from Great Field, September 1960, with the usual thermophilic moulds (up to 10 million spores/g.) and thermophilic actinomycetes (up to 100 million spores/g.), and when this hay was disturbed a cloud of white dust rose into the air.

Preliminary immunological tests

In a search for evidence connecting moulding of hay with its ability to cause Farmer's Lung disease, precipitin tests were made in agar gels with crude hay extracts against sera from Farmer's Lung patients (kindly supplied by Dr C. J. Fuller, Royal Devon and Exeter Hospital, and Drs J. Pepys and R. W. Riddell, Brompton Hospital, London). Extracts were made in a laboratory blender from 5 g. chopped hay in 200 ml. phenol saline (NaCl, 8·5 g.; phenol, 4 g.; water, 1000 ml.) and straining through muslin. Tests were done on 2 × 2 in. glass slides, with the

crude extract placed in the holes in the agar (1·5% agar, 0·02% thiomersalate, 0·85% NaCl) at the same time as the serum (Mansi, 1958).

Preliminary tests showed that sera from Farmer's Lung patients formed two (1 to 3) clear, but rather weak, precipitin bands with extracts from three batches of Farmer's Lung hays (H 32, H 44, H 55; described by Gregory & Lacey, 1963*a*), and with extracts of experimental hays E and R of 1959 described above. Bands were not formed with extracts of batches N and L of 1959. Sera from control patients did not form bands with any hay extracts.

In 1960 crude extracts were made of samples of W, W2 and Y during the moulding process at different stages and tested against serum from a person with Farmer's Lung and a control human serum. As soon as the actinomycete count and pH value had increased, W and W2 formed two precipitin bands in tests with the Farmer's Lung serum (Fig. 1*a*). Y produced one band which was rather diffuse and crossed the bands formed by W and W2, so is considered to indicate an antigen unrelated to those in W. As already pointed out, Y was not a good control hay as it had heated more than usual during maturation. Some samples of V produced bands similar to those produced by W, but the 3 early samples of X did not. Tests were also done during the moulding of pile S, and bales Q, in 1960. S also produced two precipitin bands, which were first seen in extracts made 3 days after piling; extracts of Q never produced bands and bands were never produced in tests with the control serum. Tests with other sera during the season gave general confirmation of these results. The tests were discontinued in 1961 because work with purified extracts was in progress at the Brompton Hospital. Evidently there are antigens in mouldy hay that react with sera from Farmer's Lung patients (Pepys, Riddell, Citron & Clayton, 1962), and these antigens appear as early as 3 days after piling (Batch S).

DISCUSSION

Hay while drying in the field carries a range of micro-organisms, some epiphytes, some airborne and some derived from the soil. Saprophytes, such as Cladosporium and Alternaria, occur in quantity. The diversity of propagules present suggests that different types of microflora can develop later according to the conditions that prevail in bale or stack at any time.

Experimental baled hay. From 1959 to 1961 the microflora of 22 batches of hay made at different moisture contents was studied (Table 4). Although there was wide variation between the different batches, and sometimes between samples from the same bale, a type of flora could be distinguished characteristic of hays that had become hot because the grass was too wet when baled. Typical examples were E (1959) and W (1960) with 42 and 46% moisture, respectively.

Batch W provided the clearest sequence of microbial succession during moulding of a hay of the Farmer's Lung type, because it was wetter and was more uniform than A and F (1961). After baling at 46% water content the temperature of the bale at once began to increase and in 24 hr. reached a temporary 48° maximum. The temperature then fell and reached a temporary 40° minimum at about 48 hr. after baling. During this time *Mucor pusillus* had evidently been developing, and its spores began to appear in the samples blown at 48 hr., reaching their maximum by about the 11th day. This temperature minimum was followed by a further rapid

Table 4. *Characteristics of groups of experimental hays*

Hay type	Batch	Water content at baling or stacking (%)	Max. temperature reached (°C)	pH range (after 14 days)	Maximum spore count (millions/g.) by cascade impactor: Fungi	Maximum spore count (millions/g.) by cascade impactor: Actino-mycetes + bacteria	Reaction to sera of Farmer's Lung patients	Notes
Very mouldy bales	E, R, W	40–46	60–70	6·8–7·6 (W)	9–94	370–680	E+, R+, W+	Mainly thermophilic moulds, e.g. *Absidia* spp., *Aspergillus fumigatus, Humicola lanuginosa, Mucor pusillus,* and actino-mycetes, e.g. *Micromonospora vulgaris*
Variable, but very mouldy bales	X, V, A, F	26–39	45–60	5·8–7·4	10–36	42–240	V+, X−	Thermophilic organisms as above
Mouldy bales	N, D, Y, G	20–28	35–46	5·2–6·3 (Y, G)	12–60	3–10	N−	Mainly mesophilic micro-organisms, e.g. *Aspergillus glaucus*
Good bales	L, P, B	15–17	22–26	5·9–6·0 (B)	0·1–0·7	0·4–2	.	Limited growth of mixed, mainly meso-philic micro-flora
Wet stack (acid brown core)	SA	30	63	4·5–5·6	1	1·6	.	See Table 3; Fig. 3*a*, 3*b* and 4
Wet stack (mouldy regions)	SA	30	63	6·1–7·8	21	280	.	See Table 3; Fig. 3*a*, 3*b*, 4
Good stack	SB	15	22	5·9–6·1	6·4	0·8	.	Limited growth of mixed, mainly meso-philic micro-flora
Wet hay in piles about 1 m. high × 1 m. diameter	S, Lu	Lu, 44 S, 65	48 52	. .	10 20	10 89	S+	.
Hay wetted in field	Gn, Q, T1, T2	17–26 + rain	30–45	.	13–21	2–210	Q−	Mainly mesophilic micro-organisms, e.g. *Cladosporium* sp., *Trichothecium roseum*

rise to 57° by the 3rd day, which was retained until the 6th day. During this temperature rise sporulation by *Mucor pusillus* was increasing. Spores of Absidia, (mainly *A. ramosa*) appeared by the 4th day and reached their maximum by the 12th day.

On the 6th day, when the temperature of the bales was declining, spores of actinomycetes able to grow at 60° (mainly *Micromonospora vulgaris* and *Thermopolyspora polyspora*) began to appear, and reached their maxima on about the 11th day. A group of actinomycetes able to grow at 40° appeared by the 8th day and also reached their maximum on the 11th day. At the same time, 2 other thermophilic moulds (*Humicola* (*Thermomyces*) *lanuginosa* and *Aspergillus fumigatus*) appeared and reached their maxima by about the 16th day.

As the mass cooled to about 50° by the 14th day *Aspergillus glaucus* and *Paecilomyces variotii* appeared, as well as numerous actinomycetes able to grow at 25°, and smaller quantities of *A. nidulans* and Penicillium (mainly *P. piceum*?). At this stage, before the mass had dried out, mites, especially *Acarus siro*, developed in vast numbers and microscopic examination of dust from the hay showed cast exoskeletons and faecal pellets stuffed with fungal spores. The foraging of these mites may account for the fewer mould spores in some of the later samples.

After the bales cool to ambient air temperature, microbial changes are slow. The hay reaches equilibrium with the air at about 15 % water content, and the mites disappear. There is some evidence that *Penicillium* spp. increase after some months' storage, and *Hemispora stellata* may also develop during the autumn.

Batches baled at lower water content (e.g. A at 30 % and F at 35 % in 1961) were irregularly dried. Patches rich in actinomycetes, resembling typical Farmer's Lung hays, existed a few centimetres from much drier parts which moulded with *Aspergillus glaucus* even in the 1st day, and the sequence was confused. But the early development of the thermophilic phycomycetes, *Mucor pusillus* and *Absidia* spp., during the phase of increasing temperature was confirmed. A characteristic thermophilic bacterial flora consisting almost entirely of *Bacillus licheniformis* developed in bales of the A series after 10 days.

Bales with less moisture such as N, T2 and D (1959) and Y (1960), all with about 25 % water, also moulded, but at a lower temperature and with mesophilic moulds such as *Aspergillus glaucus* and relatively few actinomycetes.

Hays of all these groups contrasted strongly with good hays, typified by L (1959), P (1960) and B (1961), with 16, 17 and 15 % water, respectively. In each of these the microflora was sparse and more mixed in species composition than in self-heated bales.

Stacked and baled hays compared (1961). The stacked hays, SA and SB of 1961, provided an interesting comparison with the bales A and B made from the same hay in wet or dry condition, respectively, and with the bales of previous years. The dry stack SB behaved essentially as the dry bales of hay B, but the wet stack SA developed quite differently from the wet bales. The large central mass of this stack became much hotter than the corresponding bales and remained hot for a long time. The hay in the centre became brown and acid with a pleasant tobacco-like odour. There was little moulding, but spore-forming bacteria, mainly *Bacillus licheniformis*, were numerous.

In the wet stack a mantle of mouldy hay developed outside the core of brown hay.

Sequential samples of SA were taken from the centre of the stack, and the results describe the development of the brown core and not of the mouldy layer outside. After the stack had been cut open in the autumn it was clear that brown hay could also become mouldy. Some of this mould developed where rain had entered the stack, other mouldy material was in thin strata between masses of good brown hay and may have been associated with particularly damp regions of the stack.

Self-heating of hay. Several phases occur during self-heating of hay, although the sequence of events is not entirely clear. Cohn (1890) established that malt sprouts heat in two stages; the first stage reflects plant-enzyme activity and stops at 40°, and the second, caused by mesophilic and thermophilic micro-organisms, stops at 65°. Hay can also show an initial rise followed by a slight drop in temperature (Figs. 1, 2*a*, 3*a*), and it seems probable that moist hay starts to heat in the same way as does respiring malt. By contrast, dead plant material will heat up steadily when wetted, the whole temperature rise according to Miehe (1930) being caused by micro-organisms.

There is no reasonable doubt that the rise in temperature is mainly caused by micro-organisms (Miehe, 1930). Temperatures of 60° can easily be reached in bales, and 65° in stacks. Stacks can become much hotter, especially when large and wet, but such temperatures cannot be attributed solely to activity of micro-organisms, which, except for bacterial spores, will be killed by short exposure to 80° or above. Heat production in the outer parts of the stack by continued microbial growth probably prevents loss of heat from the centre. This may sustain or accelerate the purely chemical reactions that must be responsible for rise of temperature above the range 65–75° (Truninger, 1929; Miehe, 1930). Nevertheless, the possible importance of micro-organisms in predisposing the hay to chemical heating must not be overlooked.

Damp bales tend to heat less than damp stacks. Possibly hay in bales is better aerated than hay in stacks and this better aeration is the cause of heat loss in excess of heat gained by the improved growth of aerobic micro-organisms, especially the fungi and actinomycetes which are so characteristic of bales that have moulded as a result of wet baling.

The biochemical changes during the early stages of hay-making are usually caused by plant enzymes. An increase in soluble-N on wilting is caused by proteolytic activity in the grass which continues until the moisture content falls below 40%; conditions of moist wilting particularly favour continued enzyme action (Kemble & Macpherson, 1954). After stacking SA, the soluble-N continued to increase for 2 days until well above the values for the bale samples A, suggesting that proteolysis continued in the stack, which remained moist, but not in the bales, which had dried out.

Volatile-N increased more in the stack SA than in the bales, possibly because greater proteolysis in the stack produced more amino acids for deamination. Volatile-N increased soon after baling or stacking (W, 1960; A and SA, 1961), and was correlated with actinomycetes growing at 60° and with bacteria growing at 60°. In silage, extensive ammonia production usually occurs only in the presence of micro-organisms (Kemble, 1956; Macpherson *et al.* 1957), but Brady (1960, 1961) reported that plant enzymes produced some ammonia.

When self-heated hay becomes acid, conditions resemble those in silage; the acids come from carbohydrate breakdown (Firth & Stuckey, 1947). In bales a

well-insulated core of brown hay has less opportunity to develop than in a stack; whereas SA samples reached as low as pH 4·5, the samples from the A bales did not decrease below 5·5; when mould developed in the bales the pH value increased and the sugar content fell, but because of better aeration and quicker drying than in the stack (which reached pH 7·8 in the very mouldy region) the highest pH was only 6·7. The W (1960) bales clearly showed a silage-like trend during the first 6 days, with the pH value decreasing from 6·5 to 5·5 before increasing to 7·6.

The acidic conditions in silage are usually associated with many lactobacilli (McDonald *et al.* 1960) but the SA samples with high acidity contained very few lactobacilli; the predominant bacterial species was *Bacillus licheniformis*, which can grow vigorously between 35° and 55° and can form acid from carbohydrates under anaerobic conditions. *B. licheniformis* was also predominant in the A bales at the peak of the heating period.

A heated hay is brown, and the intensity of brown colour correlates well with the degree of heating (van der Schaaf, Deijs & Bosch, 1954). When a brown hay has a large pH value, moulding has occurred (Truninger, 1929), as in some late SA samples (Table 3), and changes from heating cannot be distinguished from those of moulding. When we incubated sterile hay for 14 days at 60° in the laboratory, the pH value decreased from 6·0 to 5·1.

Thus, effects occur from plant enzymes, the growth of mesophilic and thermophilic micro-organisms, and probably from purely chemical reactions; all of these have to be considered in an understanding of the mechanism of moulding hay. Nevertheless, when dealing with bales some general trends have been observed. There seems ample justification for regarding the water content of the herbage at time of baling as the most critical factor which determines the subsequent microbial succession and the degree of heating attained.

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THE SOCIETY FOR GENERAL MICROBIOLOGY

The Society for General Microbiology held its thirty-eighth General Meeting in the University of Nottingham, on Thursday, Friday and Saturday, 19, 20 *and* 21 *September* 1963. *The following communications were made:*

SYMPOSIUM: 'ANTIBIOTICS: STRUCTURE AND FUNCTION'

Structure and Activity in the Actinomycins. By E. F. GALE (*Sub-Department of Chemical Microbiology, Department of Biochemistry, University of Cambridge*)

The actinomycins are a family of antibiotics all of which contain a three-ring chromophore attached to two cyclic peptides (Waksman, S. A., Katz, E. & Vining, L. C. (1958), *Proc. nat. Acad. Sci., Wash.* **44**, 602). The chromophore possesses a free amino group; modification of this group markedly decreases and may abolish biological activity. The various actinomycins differ in the amino acid composition of the cyclic peptides. Addition of a growth-limiting concentration of actinomycin to an exponentially-growing culture of *Staphylococcus aureus* results in immediate cessation of RNA synthesis; DNA and protein syntheses are inhibited after the cells have been exposed to the antibiotic for 15–20 min. Fermentation and respiration are unaffected. Treatment of *Bacillus cereus* cultures with sub-lethal concentrations of actinomycin results in production of long forms. The effects of the antibiotic on the syntheses of protein, RNA and DNA can be annulled by the simultaneous addition of DNA, but not RNA, preparations. Treatment of transforming DNA with actinomycin markedly reduces the transforming activity of the preparation. The antibiotic inhibits DNA-dependent DNA polymerase and DNA-dependent RNA polymerase but not RNA-dependent RNA polymerase. DNA-dependent RNA polymerase is approximately ten times more sensitive to inhibition than DNA polymerase; in both cases the degree of inhibition is determined by the relative amounts of DNA and actinomycin present (Kirk, J. M. (1960), *Biochim. biophys. Acta*, **42**, 167; Hurwitz, J., Furth, J. J., Malamy, M. & Alexander, M. (1962), *Proc. nat. Acad. Sci., Wash.* **48**, 1222; Elliott, W. H. (1963), *Biochem. J.* **86**, 562). Complex formation between DNA and actinomycin can be shown by a shift in the spectrum of the latter on the addition of DNA, by an increase in viscosity of DNA solutions on addition of actinomycin, and by migration of antibiotic and DNA together in an electric field (Kirk, J. M. (1960), *Biochim. biophys. Acta*, **42**, 167). There is a strong correlation between the ability of the various actinomycins to complex with DNA and their inhibitory action on the growth of *Bacillus subtilis*, on the division of HeLa cells, and on the cell-free bacterial RNA polymerase (Reich, E., Goldberg, I. H. & Rabinowitz, M. (1962), *Nature, Lond.* **196**, 743).

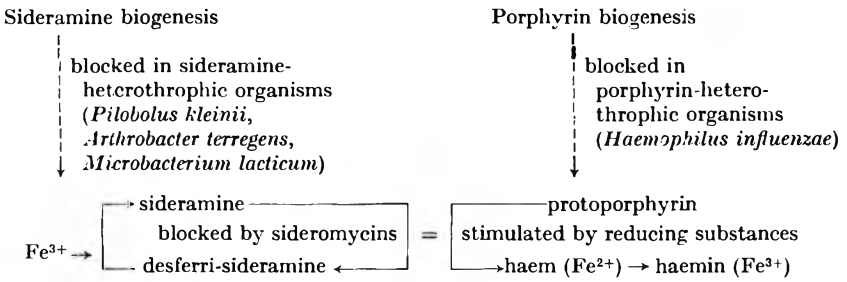
The actinomycin-DNA complex is readily dissociable in low concentrations but X-ray diffraction studies do not show the changes that would be expected if intercalation of the antibiotic between base pairs of the DNA helix had taken place (Hamilton, L. D., Fuller, W. & Reich, E. (1963), *Nature, Lond.* **198**, 538) as has been shown in the case of proflavine which has similar properties (Lerman, L. S. (1961), *J. mol. Biol.* **3**, 18). Binding of actinomycin by DNA is dependent on the presence of guanine residues in the polynucleotide. The amount of antibiotic bound by DNA, and the consequent inhibition of polymerase primed by that DNA, increases with the amount of guanine in the DNA (Goldberg, I. H., Rabinowitz, M. & Reich, E. (1962), *Proc. nat. Acad. Sci., Wash.* **48**, 2094). The amount of actinomycin bound is greatest when the DNA is in the native helical conformation. RNA synthesis can be primed by polydeoxyribonucleotides containing two bases; proflavine inhibits synthesis primed by dAT or dGC while actinomycin affects only the latter (Gold-

berg, I. H., Reich, E. & Rabinowitz, M. (1963), *Nature, Lond.* **199**, 44). Hamilton, L. D., Fuller, W. & Reich, E. ((1963), *Nature, Lond.* **198**, 538) propose that the antibiotic becomes hydrogen-bonded to deoxyguanosine residues in the DNA helix. The complex then becomes stabilized by further interaction between the cyclic peptides of actinomycin and phosphate groups of the nucleotides.

Iron-Containing Antibiotics and Growth Factors. By W. KELLER-SCHIERLEIN (*Zürich*)

In the last decade a new group of iron-containing natural products has been found in cultural fluids of many micro-organisms. We call these substances siderochromes (Gr. *sideros* = iron). Some are highly active growth promoters for micro-organisms as, for examples, ferrichrome and related substances from fungi, the ferrioxamines from actinomycetes, the 'terregens factor' from a bacterial strain, and mycobactin from an acid-fast organism, and are called sideramines. A second subgroup of the siderochromes, the sideromycins, are biologically characterized by a competitive antagonism to most of the sideramines; they are highly potent antibiotics. The best investigated sideromycins are albomycin (probably identical with grisein) and ferrimycin A; others are known which have neither growth-promoting nor antibiotic activity. The only substance of this third subgroup to be isolated in a pure state, is ferrichrome A, a side component of the production of ferrichrome by the fungus *Ustilago sphaerogena*.

The biological function of the sideramines has been elucidated to some extent. Independently, Zähner & Burnham came to the conclusion that these highly active and widespread compounds play a fundamental role in the enzymic incorporation of iron into the porphyrin skeleton. Based on his own and on Burnham's experiments, Zähner gives the following scheme for the sideramine action:



The chemical structures of sideramines of different origin may vary to an unexpected extent. The common structural feature is the presence of 3 hydroxamic acid groups as the iron binding centres. In ferrioxamine B, the major constituent of the ferrioxamine mixture from aetinomycetes, these groups are located in a long chain built up of 3 components of 1-amino-5-hydroxylamino-pentane, 2 components of succinic acid and one acetic residue. This structure has been proven by total synthesis (Prelog & Walser). Crude ferrioxamine mixtures contain a series of *ca.* 8 minor constituents. The structures of 6 of them have been determined and are closely related to that of ferrioxamine B.

A rather different structural arrangement is realized in the fungal sideramines. The hydroxamic acid groups are located in side chains of a homodetic cyclohexapeptide ring which is built up of 3δ-*N*-hydroxyornithine residues and 3 neutral amino acid residues (glycine or serine). The amino acid sequence in the ring is still unknown.

The carboxylic acid residues taking part in the hydroxamic acid groups are acetyl groups in ferrichrome (Emery & Neilands), ferricrocine and ferrichrysine, cis- and trans-5-hydroxy-3-methyl-2-pentenoic acids in ferrirhodine and ferrirubine respectively, and 3-methylglutaconic acid in ferrichrome A. Coprogen, a sideramine from several penicillium strains, is somewhat apart. The carboxylic acid moiety is the same as in ferrirubine, but except for δ-*N*-hydroxyornithine there are no other amino acids present.

An iron-complexing growth factor from *Mycobacterium phlei*, mycobactin, has been in-

vestigated by Snow. This substance contains only 2 hydroxamic acid groups. The third iron binding centre is a phenolic hydroxyl in conjunction with a nitrogen or oxygen atom of an oxazoline ring. Although ferrioxamines and the fungal sideramines can replace one another in most of the biological tests, the activity of mycobactin seems to be limited to the acid-fast organisms.

The structures of the biological antagonists of the sideramines, the sideromycins, have not been fully determined. But what has been done up to now gives a good picture of their chemical relation to the sideramines. The iron complexing part of the ferrimycin A molecule is the same as in the ferrioxamines. To the basic end of the ferrioxamine chain an aromatic acid, 3-amino-5-hydroxybenzoic acid, and a hitherto unknown residue of mol.wt. *ca.* 200 are connected.

The other sideromycin albomycin, whose chemical investigation has been advanced to some extent by Mikeš & Turková, seems to be more related to the fungal sideramines, since *δ*-*N*-hydroxyornithine and serine could be isolated from hydrolysis mixtures. A further hydrolysis product from albomycin is 3-methyluracil. Here again an unknown part of the molecule remains to be determined.

The desferri-sideramines are the most powerful iron complexing agents with complex stability constants *ca.* 10^{30}–10^{32} (Schwarzenbach). Other biologically important metal ions are bound relatively weakly. They are not toxic in animals or men. Desferri-ferri-oxamine B is the first satisfactory therapeutic agent capable of removing pathological iron deposits from the body in hitherto lethal diseases like haemochromatosis and siderosis.

The Ostreogrycins—a Family of Synergistic Antibiotics. By E. Lester Smith

The antibiotics produced by *Streptomyces ostreogriseus* and related organisms are remarkable in many ways. Members of this large family were isolated independently in at least five laboratories, in U.S.A., U.K., Belgium and Japan. They fall into two groups such that any member of one group strongly potentiates the antimicrobial action of any member of the other group. The first of these groups comprises the antibiotics Ostreogrycin B (also known as E. 129B, PA. 114B or Mikamycin B; present in Streptogramin), Staphylomycin S and the minor factors Ostreogrycins B_1, B_2 and B_3. The previously known antibiotic Etamycin (Viridogrisein) also falls into this group, making a total of 6.

The second group, regarded for convenience as the potentiated factors, comprises Ostreogrycin A (also known as E. 129A, PA. 114A or Staphylomycin M1; present in Streptogramin), Ostreogrycin G, Mikamycin A and the minor factors Ostreogrycins C, D, and Q and Staphylomycin M2; this group contains 7 antibiotics, or perhaps only 6, since Staphylomycin M2 may be identical with one of the Ostreogrycins.

The ostreogrycins are conveniently extracted from fermentation liquors with ethyl acetate, and the crude antibiotic can be precipitated with petrol from the concentrated extract. Part of the factor A can be separated by crystallization from ethyl acetate. The B factors are weakly basic and can be removed with ion exchange resins or by extraction with aqueous acid from organic solvent solution; they crystallize readily from aqueous alcohol. The separate factors have been obtained by prolonged counter-current fractionations. The same method, or partition chromatography, has been used to purify Ostreogrycin G, which has not been crystallized.

Most of the laboratories concerned have published work on the chemistry of these compounds, but the major contribution has been by Lord Todd and colleagues at Cambridge. The potentiators all contain 3-hydroxypicolinic acid linked to a cyclic peptide of 6 or 7 aminoacids, the macrocyclic ring being closed by lactonization with the hydroxyl of threonine. Except for Etamycin they differ from one another by a single amino acid. Ostreogrycins A and G are closely related complex unsaturated macrocyclic compounds of unique constitution, not yet fully elucidated.

Ostreogrycins A and G show similar *in vitro* activities against *S. aureus* and other pathogens, but the antibacterial activity of each is increased phenomenally by addition of

a potentiating factor; activity increases to a flat-topped maximum at approximately 1:1 proportion by weight. However, *in vivo* mouse protection tests show Ostreogrycin G to be at least 7 times as effective as A when both are optimally potentiated by Ostreogrycin B. Thus the G+B mixture is a potent antibiotic combination, of low toxicity moreover. Nevertheless, a combination of high cost and complexity of manufacture have militated against its use in medicine.

It seemed possible that the strong potentiation might be due to the two antibiotics interfering with different essential metabolic activities of the pathogenic organism, but published work does not support this idea. Vazquez, studying the mode of action of crude Streptogramin, and Yamaguchi, studying that of the Mikamycins, agree that incorporation of labelled amino acids into protein is inhibited, whereas incorporation into RNA is slightly enhanced in *S. aureus* and some other organisms. The Japanese work showed Mikamycin B to be slightly effective, A more so and A+B highly effective, in line with their bacteriostatic potencies. Chloramphenicol behaves in exactly the same manner, yet it shows no potentiation nor even a simple additive effect, with Mikamycin B or Streptogramin.

Structure and Function in the Penicillins and Cephalosporins. By M. H. Richmond and J. F. Collins (*National Institute for Medical Research, Mill Hill, London, N.W. 7*)

The penicillins cause inhibition of cell-wall mucopeptide synthesis in sensitive organisms, presumably by inhibition of an essential enzyme. The *N*-acylated derivatives of 6-aminopenicillanic acid have the highest activity, and in these compounds the essential functional groups are the carbonyl group in the acyl side chain, the carbonyl group and nitrogen atom that form the β-lactam bond, and the free carboxyl group. The antibiotic activity of penicillin is very sensitive to changes in the molecular structure which affect any of these groups, or alter their relative configurations. The fused ring system forms a rigid structure with the β-lactam bond in a distorted and reactive state, and the carboxyl group is sterically hindered from rotation by one of the gem-di-methyl groups. Only the acyl side chain appears to have freedom to take up varying configurations, though complete rotation about the N ring-C bond does not appear possible. X-ray analysis of benzyl- and phenoxymethyl-penicillin has shown that in the crystal the amide link is in the planar trans form, while the side chains are folded to make the molecules assume a curled configuration (like a letter C). All but one of the hydrogen-bonding groups, including the β-lactam bond, are exposed on one face of the molecule. Interaction between penicillin and an enzyme would be expected to occur between these groups in penicillin and the protein, bringing the β-lactam bond close to the surface of the enzyme. The β-lactam bond is readily split by many reagents, often giving a derivative of penicilloic acid as the product. If this type of reaction occurred between penicillin and the active site, a covalently bound complex would be formed which would inhibit the enzyme. The similar distribution of the hydrogen bonding groups in penicillin and on one face of *N*-acetylmuramic acid suggests that penicillin inhibits mucopeptide synthesis at some enzymic step involving an *N*-acetylmuramic acid derivative as a substrate.

Among the changes in penicillin that lead to impaired activity, the loss of the carbonyl group in the side chain reduces antibiotic activity several thousand-fold. When this group is present, its conformation appears to be important for antibiotic activity against Gram-positive organisms, though against Gram-negative organisms the nature of the acyl side chain is more important. The variation of activity with different side chains may be accounted for partly by the ability of this carbonyl group to assume an active configuration plus the stability of the molecule in this form.

The cephalosporins appear to act in the same way as the penicillins, though the nucleus of the molecule is different. However, the hydrogen bonding groups significant in penicillin are also present in cephalosporin C, but their relative spatial configurations have not been precisely established. The carboxyl group is not as sterically hindered as that in penicillin, while the acyl side chain may be able to rotate. This freedom may account for the low antibiotic activity of cephalosporin C, and also for the higher activities of *N*-acyl deri-

vatives of 7-amino-cephalosporanic acid, in which the side chain can assume more favourable configurations.

Antibiotic activity of the penicillins and cephalosporins against penicillinase-producing organisms is complicated by the effect of the antibiotic in inducing the enzyme and acting as a substrate. In general, induction ability and antibiotic efficiency are correlated, while the rate of hydrolysis of penicillins by the enzyme is determined largely by the side chain, though individual penicillinases show considerable variation in their properties. Cephalosporin C is poorly attacked by penicillinase (though other derivatives are more susceptible), but it is rapidly destroyed by the cephalosporinases produced by several Gram-negative strains of organisms. These enzymes have low activity against benzylpenicillin.

Some Antibiotics which act as Analogues of Metabolites. By K. McQuillen (*Sub-Department of Chemical Microbiology, Department of Biochemistry, University of Cambridge*)

Analogues may interfere with the formation of small metabolites or complex end-products such as proteins and nucleic acids. In each case the sensitive reaction may be universal or may be restricted to micro-organisms. If the former situation obtains, the invader must be more susceptible to the antibiotic than is the host (perhaps because of relative importance of the reaction) or the site of the reaction must be less accessible in the host than in the invader. Substances which prevent synthesis of nucleotides (e.g. azaserine, DON-glutamine analogues) or which are themselves amino acid, base or nucleoside analogues are not, in general, effective in the treatment of infectious diseases. However, some may have applications in cancer therapy.

Antibiotics which are analogues of metabolites specific to the invader are more useful. Components of bacterial cell-wall mucopeptides are in this category and several successful antibiotics interfere with wall formation: some have been demonstrated to be analogues. D-Cycloserine (oxamycin) is an analogue of D-alanine which occurs in wall mucopeptide. Inhibition of growth by cycloserine is competitively antagonized by D-alanine and the antibiotic inhibits the formation of D-alanine from its isomer and also the conversion to D-alanyl-D-alanine which is subsequently built into a cell-wall precursor. Cycloserine inhibition of formation of this precursor is non-competitively antagonized by the dipeptide. It is likely that the antibacterial action depends on cycloserine's being an analogue of a metabolite specific to bacteria and absent from the host. Penicillin (see paper by Richmond and Collins, this symposium), bacitracin, and vancomycin also act at some stage in mucopeptide biosynthesis but the reactions concerned and/or whether these substances are all analogues have not been established.

Some antibiotics inhibit synthesis of protein without preventing the formation of nucleic acids or the activities of many other enzyme systems. Two, at least, appear to act as analogues and their site of action has been localized biochemically. They are amino acid derivatives of amino-ribonucleosides—puromycin and homocitrullylamino adenosine. After amino acids are activated by reaction with ATP they are added to the terminal adenosine of specific transfer-RNA molecules. The complexes then interact with ribosomes where the amino acids are positioned appropriately before being joined by peptide bonds to growing polypeptide chains with elimination of transfer-RNA. Puromycin can be mistakenly added on and cause premature termination of a chain—presumably because of its structural resemblance to the amino acid–adenosine end of the normal metabolite. The structural requirements for antibiotic activity have been studied extensively.

The observations mentioned here are not the work of the writer but of many other people and a few key references are given below:

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NATHANS, D. & NEIDLE, A. (1963). Structural requirements for puromycin inhibition of protein synthesis. *Nature, Lond.* **197**, 1076.

Some Relations between Chemical Structure and Antifungal Effects of Griseofulvin and its Analogues. By A. RHODES (*Glaxo Research Ltd., Sefton Park, Stoke Poges, Bucks*)

Griseofulvin is firmly established in many countries as an oral antibiotic for treatment of fungal diseases in both medical and veterinary practice: it is also effective in crop protection as a systemic 'fungicide' for control of specific plant diseases.

Griseofulvin affects specifically the morphogenesis of fungi with chitinous cell walls, causing production of characteristic spirally curled and malformed hyphae. These peculiar effects may be due to interference with biosynthesis of the chitinous cell wall. Alternatively, incorporation of griseofulvin into a modified chitin might account for formation of spirally curled hyphae with weakened cell walls; if this were so, differences in sensitivity to griseofulvin among chitinous-walled fungi might then reside in differences of 'combining affinity' between the fungus cell and griseofulvin.

The antibiotic is not translocated within fungal hyphae, but its systemic action in plants and animals may be supremely important for eradicant action. Whenever griseofulvin can be used so as to permeate tissue continuously at concentrations of little more than 1 μg./ml. before invasion by a pathogenic fungus, then the griseofulvin-containing tissues may be expected to exert a fungistatic action by forming a barrier to penetration by griseofulvin-sensitive fungi. Eradication of the fungus is to be expected if the perimeter of this antibiotic barrier is so placed as to confine the pathogenic fungus to host tissue that may be subsequently shed.

Griseofulvin or 7-chloro-4,6,2′-trimethoxy-6′-methylgris-2′-en-3,4′-dione is only one of four possible stereo-isomers. The sterically less-favoured configuration of the natural isomer, in which the bulky 3-carbonyl and 6′-methyl groups are cis-related, is designated by the prefix (dd′)- referring to the two asymmetric centres 2 and 6′ respectively.

Elimination of the 6′-asymmetric centre diminishes antifungal activity but change from *d* to *l* configuration at both asymmetric centres simultaneously, or at each separately, completely prevents this biological action.

Diverse structural features contribute to the antifungal activity of griseofulvin. Intact ether linkages are essential; susceptibility of all three methoxyl groups to enzymic breakdown yielding mono-demethylgriseofulvins of low activity may therefore account for the gradual degradation of griseofulvin observed in fungi, plants and animals. Biological activity of the antibiotic also requires a free 5-position, an intact 5-membered B ring containing the reactive oxo-group at position 3, and a polar group at position 4′ in ring C. The favourable influence of the chlorine atom at the 7-position in ring A can be equalled only by replacement with fluorine.

The antifungal activity of griseofulvin is enhanced by homologous alkyloxy group replacements in the 2′-position or by substitution of halogen, benzyl or certain alkyl groups in the 3′-position of ring C but increased activity against plant pathogenic fungi predominates over improved action against dermatophytes. Association of the more active substituent groups at the 3′-position with the more active replacement groups at the 2′-position shows a potentiated response in several analogues but antifungal actions *in vitro* and *in vivo* are affected in different degree. Substitution at the 3′-position, although enhancing *in vitro* antifungal activity, may fail to sustain this advantage by interfering with processes that affect systemic action in plants and animals. These results are compatible with a suggestion already published that for each similar group of griseofulvin analogues there may be an optimum high oil-water partition coefficient, both for increased activity *in vitro* and for systemic antifungal action.

Utilizing this knowledge, it is possible to synthesize analogues more effective than griseofulvin against certain plant diseases but none has proved superior to griseofulvin for control by oral therapy of the fungi that cause disease in hair, skin and nails of man.

ORIGINAL PAPERS

Potassium and Rubidium Uptake by *Staphylococcus aureus* and *Escherichia coli* compared. By K. A. Wright and H. V. Wyatt (*Bradford Institute of Technology, Bradford*)

Potassium utilization by *Staphylococcus* differs from that by Gram-negative organisms (Wyatt, H. V. (1963), *Exp. Cell Res.* **30**, 56, 62). We have now used similar media and methods to compare *Staphylococcus* and *Escherichia coli* under similar experimental conditions. Net uptakes of ^{42}K and ^{86}Rb added to a medium made deficient in potassium were measured by the decrease in radioactivity of supernatants. Duplicate series of tubes contained equal total amounts of K^+ and Rb^+ including either ^{42}K or ^{86}Rb. Growth in the duplicate experiments was almost identical. Total cell crop increased with K^+ concentration and addition of Rb^+ further increased it. The efficiency of Rb^+ compared with K^+ showed similarities for both organisms. Because of different ratios of dry weight to opacity, the same K^+ supported three times more mass of *E. coli* than *Staphylococcus*. Potassium uptake was similar in both organisms, but whereas *Staphylococcus* maintained a 50,000-fold accumulation after growth ceased, *E. coli* immediately leaked K^+ and the accumulation dropped to 200-fold. With addition of Rb^+, there were similar patterns of competition between Rb^+ and K^+ with similar diphasic logarithmic growth. Depletion of supernatant K^+ was greater in both cases when Rb^+ was added (there was increased utilization of K^+ and Rb^+ was taken up as well). With *E. coli* this depletion was much exaggerated: because of the greater mass of *E. coli*, the K^+ accumulation was however similar in both organisms. Rubidium inhibited K^+ efflux at the end of growth. Rubidium efflux was similar to that of K^+. Addition of caesium further inhibited K^+ uptake. Under similar conditions, the utilization and uptake of potassium and rubidium by *E. coli* and *Staphylococcus* showed many similar patterns. The differences between the two organisms were less than had been expected.

Some Effects of 3-Carboxy-2-quinoxalinylpenicillin (Quinacillin) on Bacteria *in vitro*. By D. F. Spooner and A. B. Spicer (*Biology Division, Research Department, Boots Pure Drug Company, Nottingham*)

Acylation of 6-aminopenicillanic acid (Batchelor, F. R., Doyle, F. P., Nayler, J. H. C. & Rolinson, G. N. (1959), *Nature, Lond.* **183**, 257–8) has provided a means of preparing a variety of new penicillins. Quinacillin is a new semi-synthetic penicillinase-resistant penicillin (Richards, H. C., Housley, J. R. & Spooner, D. F. (1963), *Nature, Lond.* in the Press) with some unusual properties.

Quinacillin is active against both penicillinase-producing and benzylpenicillin-sensitive strains of *Staphylococcus aureus* in low concentration (0·1–0·6 μg./ml.) *in vitro*. However, the spectrum of antimicrobial activity is atypical for a penicillin in that activity is greater against staphylococci than against other species of Gram-positive bacteria including strains of streptococci highly sensitive to benzylpenicillin. The activity against Gram-negative bacteria is very low and several species are not inhibited by concentrations as high as 10,000 μg./ml. Bio-assay of residual quinacillin after incubation with concentrated suspensions of cells has shown that, in most cases, cells of insensitive species do not inactivate the compound.

The activity of quinacillin against staphylococci is enhanced several fold as the pH value of the medium is lowered over a pH range compatible with growth. The rate of diffusion in agar of this penicillin also appears to be enhanced under acidic conditions.

Despite the high activity against penicillinase-producing staphylococci quinacillin is, at low concentrations, a poor inducer of penicillinase synthesis and, with *S. aureus* (Parker) in casein-hydrolysate medium, the 'induction constant' (Pollock, M. R. (1957), *Biochem. J.* **66**, 419–28) is much greater than with other penicillinase-resistant penicillins.

Experiments on the effect of quinacillin on the growth of staphylococci and on the induction of penicillinase synthesis are described and the mode of action discussed.

Bacteriophage Sensitivity and Biochemical Type in *Xanthomonas malvacearum*. By A. C. HAYWARD (*Commonwealth Mycological Institute, Kew*)

Xanthomonas malvacearum, the cause of bacterial blight of cotton, was isolated from collections of diseased leaves originating in the main from Africa, Australia and the United States. Isolates were differentiated into two groups differing in colony form on first isolation and in some biochemical characteristics. Type I isolates produced raised convex, viscid colonies, failed to oxidize lactose and were weakly proteolytic; type 2 isolates produced low-convex, butyrous colonies, and were lactose positive and strongly proteolytic. Representative cultures of each type were tested for their susceptibility to thirteen bacteriophages of large plaque size isolated from diseased cotton leaves (Rosberg, D. W. & Parrack, A. L. (1955), *Phytopathology*, **45**, 49). Six of the phages showed type 1 specificity, the other 7 lysed only type 2 strains at routine test dilution (10^{-5} dilution of a suspension containing 10^{10}–10^{11} phage particles per ml.). Suspensions of higher titre did not show absolute specificity: some type 1 phages gave discrete plaques on type 2 cultures of *X. malvacearum*, but type 1 cultures were rarely lysed by type 2 bacteriophages. Moribund cotton leaves or seed usually, but not invariably, proved to contain a bacteriophage to which the infecting strain of *X. malvacearum* was susceptible. Material infected with *X. malvacearum* type 1 yielded a phage active against isolates of this type from all sources, and similarly type 2-specific phages were obtained from type 2-infected material. The two types of *X. malvacearum* may occur together or in isolation in different territories, and the present known distribution of each type will be given. Other xanthomonads (30 strains of 14 species) were not susceptible to phage lysis by 7 representative *X. malvacearum* phages, although some caused inhibition of growth probably due to lethal adsorption or bacteriocin activity.

Influence of Substrate Purification and Trace Element Addition on Effects of Inoculum Size in *Aspergillus oryzae*. By A. F. MCINTOSH and J. MEYRATH (*Department of Applied Microbiology and Biology, Royal College of Science and Technology, Glasgow*)

Further to earlier reports indicating that the effects of inoculum size on growth of *Aspergillus oryzae* in synthetic media are abolished by certain trace element compositions (Meyrath, J. (1963), *Leeuwenhoek ned. Tijdschr.* **29**, 57; Meyrath, J. & McIntosh, A. F. (1963), *J. gen. Microbiol.* in the Press), it has now been shown that those effects can be reversed by a procedure known to remove trace elements. If the sugar of substrate A_1 with 40 g. glucose/l. is subjected to treatment by aluminium sulphate (Shu, P. & Johnson, M. J. (1948), *J. Bact.* **56**, 577) at a pH value of 7·0 or 8·5, the growth rate over the largest part of the growth curve is appreciably smaller with large inocula (5×10^7 washed conidia/100 ml. substrate) than with small inocula (5×10^3/100 ml.). Treatment at pH values of 5·0 and 5·5 does not cause this reversal but abolishes the 'usual' effects of inoculum size. Addition of specified traces of salts of Fe, Ca, Mn, Cu to a substrate purified with aluminium sulphate at a pH value of 7·0 does not markedly change the phenomena observed with treated unsupplemented substrate. Addition of very small amounts of Zn to a purified and trace element-supplemented substrate as above results in an inhibition of the growth rate in small-inoculum cultures as compared with no Zn or with large amounts of Zn; this inhibition is not pronounced in large-inoculum cultures.

Addition of Ca to substrate A_1 increases the 'usual' effects of inoculum size whereas Cu abolishes them.

Influence of Media Components on Respiration of *Aspergillus oryzae* at Various Stages of Growth. By J. MEYRATH and A. F. MCINTOSH (*Department of Applied Microbiology and Biology, Royal College of Science and Technology, Glasgow*)

With substrate A_4 (Meyrath, J. & McIntosh, A. F. (1963), *J. gen. Microbiol.* in the Press) the respiration of mycelium from large-inoculum cultures is very sensitive to the influence of culture filtrate. Even in the absence of any mechanical influence (McIntosh, A. F. & Meyrath, J. (1963), *J. gen. Microbiol.* **31**, vi) mycelium from young cultures when

washed with water or buffer and suspended in water or buffer shows a much smaller specific rate of oyxgen uptake than unwashed mycelium suspended in its culture filtrate. Suspending washed mycelium in its culture filtrate restores only part of the inhibition. Mycelium from later stages of growth is not nearly so sensitive towards this washing procedure.

The observed stimulation by culture filtrate cannot he due to an increased formation of enzymes during the test since linear reaction curves as for 'resting' mycelium are obtained and since prolonged contact with the filtrate, up to three hours, had no effect. The stimulation has been shown to be due almost entirely to a combined action of ammonium sulphate and the heavy metal salts of substrate A_4. The increased rate of oxygen uptake is likely to be due to a stimulation of enzyme activity by metals which might be transported through the cell membrane by complex formation with ammonium ions and replace the metals which have been leached out by washing. The latter phenomenon does not appear to occur with old mycelium.

The results are in accord with previous observations that growth-stimulating or inhibiting substances excreted by the mould (Meyrath, J. (1962), *Experientia*, **18**, 41; (1963), *Leeuwenhoek ned. Tijdschr.* **29**, 57) have to act at early stages of growth in order to have an effect on late stages. Furthermore, since various trace element combinations (McIntosh, A. F. & Meyrath, J. (1963), *J. gen. Microbiol.* [accompanying abstract]) can so profoundly alter the effects of inoculum size on growth, it is possible that these growth-stimulating and inhibiting substances are of chelating nature.

Some Observations on the Airborne Dissemination of Rumen Bacteria. By S. O. Mann (*Rowett Research Institute, Bucksburn, Aberdeen*) introduced by P. N. Hobson)

Some rumen organisms (e.g. the larger protozoa) appear to be transmitted only by physical contact between animals, but as most of the rumen bacteria develop in animals which are reared without direct contact with older animals airborne or other modes of transmission must be operative in these cases. As part of a programme to find the ways by which bacteria become established in the rumen, the atmosphere of a cow-byre was examined for rumen types of bacteria. A bead-bubbler type of apparatus (Wheeler, S. M., Foley, G. E. & Jones, T. D. (1941), *Science*, **94**, 445) was used, organisms being collected in a phosphate + sodium alginate solution (pH 6·8). Samples of air taken over a period of 2 hr. showed the presence of anaerobic bacteria. Organisms of the genera *Ruminococcus*, *Veillonella* and *Eubacterium* were found. The average number of anaerobic bacteria was 28 per 15 cu.ft. air, although once in 34 experiments numbers as high as 170 per 15 cu.ft. air were found. Strains of ruminococci gave only trace amounts of growth in the absence of rumen fluid in the culture medium; this is in accord with the requirements of strains isolated from the rumen.

An assessment of the numbers of *Streptococcus bovis* and lactobacilli was made using a slit sampler. Average counts for a number of samples were: *S. bovis*, 4 organisms per 15 cu.ft. air; lactobacilli, 2 organisms per 15 cu.ft. air. As the method of sampling would unavoidably expose the anaerobic bacteria to unfavourable conditions (a solution through which air was continually bubbled) the recoveries of bacteria might be expected to be low, so that the fact that anaerobic bacteria of the typical rumen population were found in the air suggests that airborne transmission of these organisms between old and young animals is likely, especially in the confined spaces of farm buildings.

My thanks are due to Dr P. N. Hobson for his interest and advice during these investigations and the valuable assistance of Mrs Sheila Blackburn is gratefully acknowledged.

The Effect of Streptogramin and Related Compounds on *Staphylococcus aureus*. By D. Vazquez (*Sub-Department of Chemical Microbiology, Department of Biochemistry, University of Cambridge*)

Streptogramin resembles chloramphenicol in its effect on the synthesis of protein and nucleic acids by *Staphylococcus aureus* (Vazquez, D. (1962), *Biochim. biophys. Acta*, **61**, 849). The antibiotic preparation consists of two major components *A* and *B* which are

closely related to ostreogrycins *A* and *B* and to *PA* 114*A* and B_1 (Ball, S. *et al.* (1958), *Biochem. J.* **68**, 24P; Hobbs, D. C. & Celmer, W. C. (1960), *Nature, Lond.* **187**, 598). A study has been made of the effect of the purified compounds, separately and in mixtures, on the metabolism of *S. aureus*, in an attempt to determine their mode of action and to explain the marked synergism shown by these antibiotics.

The minimum growth inhibitory concentrations of *PA* 114*A*, *PA* 114B_1 and a mixture (A/B_1 = 5/1) are 6, 10 and 0·6 μg./ml. respectively. The purified components are bacteriostatic, whereas the mixture is bactericidal. *PA* 114*A* and B_1 separately or together have no effect on oxidation of ethanol or glucose by *S. aureus*. At the relevant minimum growth inhibitory concentration *PA* 114*A* alone inhibits the incorporation of ^{14}C-glycine into the protein fraction, but has no effect on the incorporation into the nucleic acid or cell wall fractions; *PA* 114B_1 alone reduces the incorporation of ^{14}C-glycine into the protein, nucleic acid, cell wall and pool fractions, while the mixture of *PA* 114*A* and B_1 has the same effect as streptogramin or *PA* 114*A* alone. Similar results have been obtained with ^{14}C-lysine.

^{14}C-labelled chloramphenicol is taken up by whole cells and by 'ribosomal' preparations of *S. aureus* (Vazquez, D. (1963), *Biochem. Biophys. Res. Commun.* **12**, 409). This uptake is completely prevented by the presence of *PA* 114*A*. It would appear possible that chloramphenicol and the streptogramin antibiotics have a related site of action.

The Effects of Antimicrobial Agents on Ribonucleic Acid Polymerase. By M. J. Waring (*Sub-Department of Chemical Microbiology, Department of Biochemistry, University of Cambridge*)

The DNA-dependent RNA polymerase of *Escherichia coli* B has been extracted from logarithmically growing cells according to the method of Chamberlin & Berg ((1962), *Proc. nat. Acad. Sci., Wash.* **48**, 81) and an improved assay has been developed using membrane filtration. The incorporation of radioactivity from ^{14}C-ATP into an acid-insoluble form is highly dependent upon the presence of all four ribonucleoside triphosphates and a DNA primer.

The effects of a range of antimicrobial agents have been studied on the purified enzyme system and among those producing significant effects are actinomycin D and the antiprotozoal drug ethidium bromide. The results obtained with actinomycin are in agreement with those of Hurwitz, Furth, Malamy & Alexander ((1962), *Proc. nat. Acad. Sci., Wash.* **48**, 1222). Ethidium bromide has been shown by Newton ((1957), *J. gen. Microbiol.* **17**, 718) to inhibit nucleic acid synthesis *in vivo* by *Strigomonas* (*Crithidia*) *oncopelti* and by Elliott (1963), *Biochem. J.* **86**, 562) to inhibit the DNA-dependent DNA polymerase of *E. coli*. Elliott also described the spectral shift which occurs when ethidium bromide is added to solutions of DNA.

It has now been shown that ethidium bromide will form complexes with a range of DNA and RNA preparations and quantitative data on the interactions have been obtained by physical methods. The action of the drug on the purified RNA polymerase has also been studied under a wide variety of conditions and the results considered in relation to the physical data on complex formation with the object of relating the biological action of the drug to its ability to complex with DNA.

An Anti-viral Effect of Extracts of Molluscum Contagiosum. By Roy Postlethwaite (*Department of Bacteriology, Foresterhill, University of Aberdeen*)

The human skin tumour, molluscum contagiosum, has long been known to be transmissible to humans by inoculation of sterile extract filtrates (Wile, U. J. & Kingery, L. B. (1919), *J. cutan. Dis.* **37**, 431) and to contain particulate entities resembling pox viruses (Rake, G. & Blank, H. (1950), *J. invest. Dermat.* **15**, 81; Banfield, W. G. & Brindley, D. C. (1959), *Ann. N.Y. Acad. Sci.* **81**, 145). Lesion extracts have been shown to cause the development of inclusion bodies and cytopathic effects in tissue cultures but these effects

were not transmissible beyond a few passages (Chang, T. W. & Weinstein, L. (1961), *J. invest. Dermat.* **37**, 433; Neva, F. A. (1962), *Arch. intern. Med.* **110**, 720).

In this study extracts prepared from molluscum lesions failed to produce any gross cytopathic effect in monolayers of mouse embryo cells but induced in such monolayers, after exposure for 24 hr., a resistance to the development of viral plaques by subsequently added vaccinia, herpes and encephalomyocarditis viruses. Preparations from individual lesions varied in activity, the degree of which, measured by a plaque-inhibition assay, was related to the concentration of extract used. One such extract had a 50 % plaque-inhibitory titre of 1:3,900.

The inhibitory activity of extracts was sedimented by high speed centrifugation, was relatively stable at −20°, 4° and 37°, but was rapidly inactivated at 56°. Activity was weakly neutralized by patients' serum, was destroyed by treatment with ether, trypsin and pH 2 buffer but was unaffected by deoxyribonuclease.

Although non-specific in its anti-viral spectrum, the properties of the inhibitor suggest it was viral in nature rather than interferon *per se*. It is possible, however, that the inhibitory effect was mediated by the development in cell cultures of an interferon-like material under the stimulus of virus present in molluscum extracts.

The Radiobiological Analysis of a Lysogenic System—*Salmonella typhimurium* phage P 22 By B. E. B. Moseley (*Molteno Institute, Cambridge*)

Salmonella typhimurium LT22 is lysogenic for phage P22. X-irradiation (210 Kvp., 10 mA.) of a log phase culture of this strain induced the cells to form phage with consequent lysis of the cells. On plotting the surviving fraction on a log scale against dose, an unusual curve was obtained: the first part was exponential, i.e. a one-hit curve with a D37 of 7·2 Krads. At about 20 % survival it changed direction, becoming steeper, and down to less than 1 % survival had the characteristics of a 5-hit slope with a D37 of 3·6 Krads.

A non-lysogenic mutant which acted as an indicator for phage P22 was derived from strain LT22 by u.v. irradiation. On re-infection of the cured strain with phage P22 a fresh lysogenic strain was obtained. This lysogenic strain showed a survival curve similar to that for strain LT22, whereas the indicator strain showed only the one-hit inactivation. On irradiating the lysogenic strain in the resting phase, when its aptitude for induction was almost zero, the multi-hit component of the survival curve disappeared, death being caused by one-hit inactivation. Thus although death of non-lysogenic cells may be caused by single-hit damage, the death of lysogenic cells may be caused either by single-hit damage or by an apparent multi-hit effect, which however represents single-hit damage on a multiplicity of targets of higher sensitivity, the latter being responsible for induction of phage production.

Those cells which were not recovered as survivors were all induced to form phage since on plotting the incidence of induction against dose the resulting curve followed the survival curve. Thus at high doses of X-ray, induction approached 100 %.

Infectious Drug-resistance in Bacteria. By Naomi Datta (*Department of Bacteriology, Postgraduate Medical School of London*)

Infectious drug-resistance was discovered in 1959 in Japan where many Shigella strains are found to be resistant simultaneously to streptomycin, tetracycline, chloramphenicol and sulphonamides. The resistance is transmissible to Gram-negative bacilli of many different genera, and has been shown to be mediated by an extra-chromosomal genetic element or episome (see Watanabe (1963), *Bact. Rev.* **27**, 87). There are reasons for believing that infectious drug-resistance is not uncommon in Europe. It was found: (1) in strains of *Salmonella typhimurium* isolated in London in 1959 (Datta, N. (1962), *J. Hyg., Camb.*, **60**, 301); (2) in *Escherichia coli* isolated from patients in a London hospital (Datta, N., unpublished); (3) in some strains of *S. typhimurium* isolated in Holland (Manten, A., Kampelmacher, E. H. & Guinée, P. A. M. (1962), *Leeuwenhoek ned. Tijdschr.* **28**, 428,

and Datta, N., unpublished); (4) in *S. typhimurium* and *E. coli* in West Germany (Lebek, G. (1963), *Zbl. Bakt.* (*I. Orig.*), **188**, 494). In these instances resistance to different combinations of drugs is found.

There is evidence that the resistance factor (R-factor) consists of deoxyribonucleic acid (DNA) which is transmitted from one bacterium to another during cell conjugation. It is possible in some cases to separate episome DNA from host DNA by making use of their different densities, which are dependent on their base ratios (Marmur, J., Rownd, R., Falkow, S., Baron, L. S., Schildkraut, C. & Doty, P. (1961), *Proc. nat. Acad. Sci., Wash.* **47**, 972). Most species of Enterobacteriaceae have DNA with a guanine + cytosine (GC) ratio of about 50 % and so do the R-factors so far studied. The DNA of *Proteus mirabilis* has a GC ratio of 39 % with a correspondingly lower density. Extracts of DNA were made by Dr S. Falkow of the Walter Reed Institute, Washington, from a strain of *P. mirabilis* before and after infection with R-factors isolated in Japan and in London. The extracts were ultracentrifuged to equilibrium in a caesium chloride density gradient and the centrifuge tubes then photographed with ultraviolet optics. In the extract from the uninfected Proteus, a single band of u.v. absorption appeared, corresponding to the bacterial DNA, but extracts from cultures carrying R-factors showed in addition satellite absorption bands, corresponding to DNA of higher density. The Japanese R-factor appeared to consist of two distinct molecular species, whereas the London one showed a single absorption band.

Peroxidase–Thiocyanate Inhibition of Streptococci in Raw Milk. By B. Reiter, A. Pickering, J. D. Oram and G. S. Pope (*National Institute for Research in Dairying, University of Reading*)

The ability of raw milk to inhibit many species of bacteria was probably first observed by Hesse in 1894 (*Z. Hyg. InfektKr.* **17**, 238), but neither the identity nor the mode of action of the inhibitor(s) (or lactenin(s)) is yet understood. One of these lactenins has been associated with lactoperoxidase (Wright, R. C. & Tramer, J. (1957), *J. Dairy Res.* **24**, 174). We have recently confirmed that lactoperoxidase inhibited some strains of *Streptococcus cremoris* in milk but we found that it failed to do so in dialysed milk or synthetic media. The dialysable factor required for inhibition was alkali-, acid- and heat-resistant, absorbable to anionic exchange resin, and has now been identified as SCN^-. The inhibition requires peroxidase, SCN^- and H_2O_2 which is produced under aerobic conditions by the inhibitory organisms. Cell multiplication, lactic acid production in milk and O_2 uptake by resting cells were all found to be inhibited. This system of inhibition may be of wider biological interest, as it is known that SCN^- inhibits the iodide uptake of the thyroid gland (Torey, W., Taurog, A. & Chaikoff, I. L. (1957), *J. biol. Chem.* **227**, 773) and was also found to be the dialysable bactericidal agent in saliva (Zeldow, B. J. (1963), *J. Immunol.* **90**, 12). Peroxidase is present in saliva and it therefore seems likely that the system found in milk also operates in saliva.