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The Antigenic Structure of Haemophilus and Corynebacterium Species from the Human Genital Tract Claimed to be Associated with or Derived from *Mycoplasma hominis*

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SUMMARY

The antigenic structure of *Haemophilus vaginalis* (Gardner & Dukes, Edmunds, and Amies & Jones strains) was compared by means of gel-diffusion precipitin reactions with that of *H. influenzae* and *Corynebacterium cervicis* (Laughton). *Haemophilus vaginalis* (Amies & Jones) was closely related to *H. influenzae*, and formed a group with related species; *H. vaginalis* (Gardner & Dukes) and *C. cervicis* formed separate groups; but all three groups possessed common antigenic components.

These strains of bacteria are among those reported as being related to *Mycoplasma hominis*, and their antigenic relationships are thus of importance in this connexion.

INTRODUCTION

Several observations have been published tending to establish a relationship between *Mycoplasma hominis* and certain bacteria which have been described as species of the genus *Corynebacterium* (Minck, 1953; Wittler, Cary & Lindberg, 1956; Smith, Peoples & Morton, 1957; Pease & Laughton, 1962; Pease, 1962) or of the genus *Haemophilus* (Amies & Jones, 1957). Whereas in most of these cases the mycoplasma (PPLO) concerned have been identified as *M. hominis* type 1 or type 2 (Campo), the identity of the related bacteria has been problematical. One of these, described by Pease & Laughton (1962), was identical with *Corynebacterium cervicis* (Laughton, 1951, 1954). Amies & Jones (1957) identified their organism as *Haemophilus vaginalis*, but Lapage (1961) and Zinnemann & Turner (1963) considered that it was identical with *H. influenzae*, and this was supported by the serological studies of Redmond & Kotcher (1963). Pease & Laughton (1962) noted resemblances between the descriptions of *H. vaginalis* (Leopold, 1953, 1956; Gardner & Dukes, 1955; Edmunds, 1960, 1962; Lapage, 1961) and *C. cervicis*.

In the present study we have examined, by the gel diffusion technique, the antigenic relationships of *Corynebacterium cervicis*, *Haemophilus vaginalis* (Amies & Jones), *H. vaginalis* (Edmunds) and *H. vaginalis* (Gardner & Dukes), with the object of obtaining information upon the bacterial types claimed to be related to *Mycoplasma hominis*.

METHODS

Organisms. Seven strains of *Corynebacterium cervicis* (Laughton: 12EA, 9T, A9T, 16T, 18T, 25T, 26T) had been isolated in this laboratory from human genital tracts; one strain of *Haemophilus vaginalis* (Gardner & Dukes: G-D) and two of *H. vaginalis* (Amies & Jones: H689, Dingham) were provided by Dr K. Zinnemann; seven strains of *H. vaginalis* (Edmunds: GP1 to GP7 inclusive) were provided by Dr P. N. Edmunds. For comparison, five strains of *H. influenzae* (originally Pitman types *a*, *c*, *d*, *e*, *f*; capsules were not investigated), and one of *H. parainfluenzae* were obtained from the National Collection of Type Cultures.

Serology. Antigens were prepared in the same manner for rabbit immunization and for gel-diffusion tests. The bacteria were grown for 48 hr at 37° in Fildes digest broth or in the medium described by Laughton (1963); they were harvested by centrifugation and washed three times with physiological saline containing 0·5% (w/v) phenol, made up to a concentration corresponding to 3 times that of no. 9 Brown's opacity tube (Burroughs Wellcome), centrifuged, subjected to ultrasonic disintegration for periods up to 20 min. according to the degree of resistance shown, and restored, in the same solution, to the volume at which opacity was measured. Rabbits were immunized, first by two subcutaneous injections at an interval of 14 days (each 1 ml. antigen + 1 ml. Freund's adjuvant) followed by a graded series of intravenous injections of the antigen alone (0·5–2 ml.) increasing by 0·5 ml. on ten occasions over 3 weeks. After a further 10–14 days the animals were bled and re-immunized when necessary.

The gel-diffusion agar had the following composition: sodium barbitol, 7·0 g.; sodium chloride, 6·0 g.; N-HCl, 2·7 ml.; Davis agar, 12·0 g.; distilled water to 1 l.; adjusted to pH 7·4.

Gel-diffusion tests were made at 20° in a moist atmosphere and readings were taken at intervals up to 10 days.

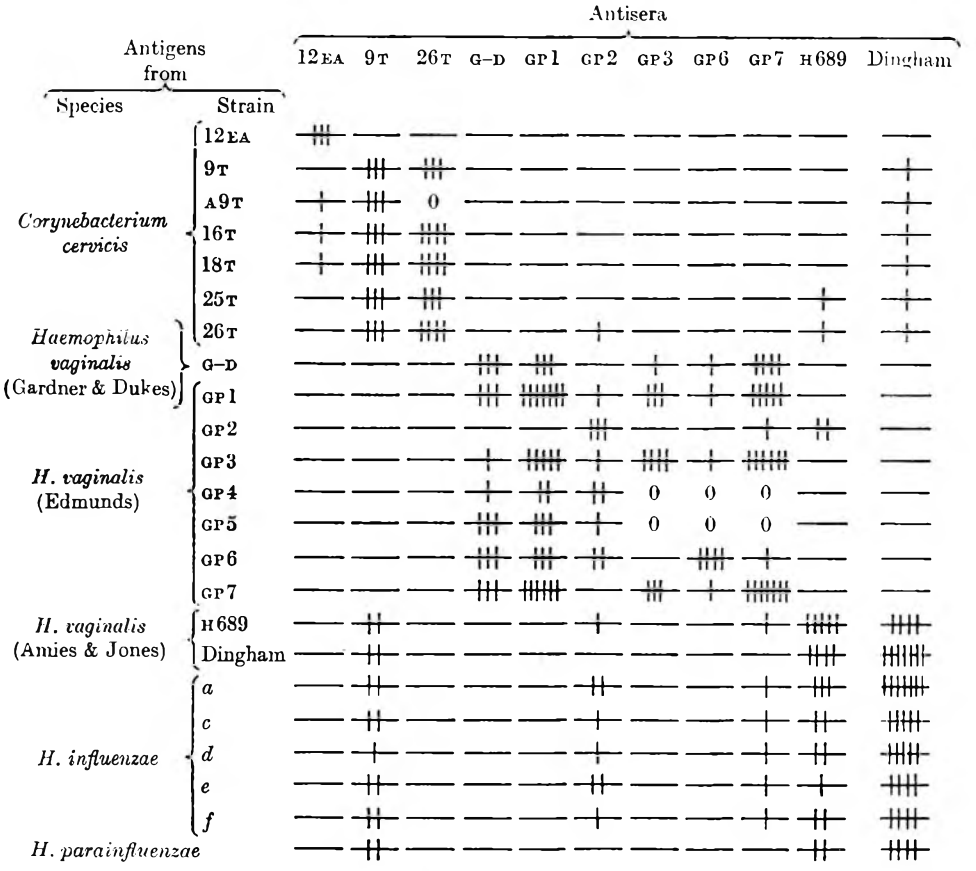
The following controls were used. Because the media used for growth contained horse serum, an excess of it (25%) was included in the agar gel to eliminate false reactions from this source. Control antigens, comprising the complete media and also, separately, laked horse red cells and Fildes digest broth, were included in each set of tests, at the usual concentrations and in 1/2, 1/4, 1/8, 1/16, 1/32 dilutions. Whole bacteria were also tested as control antigens to eliminate the possibility of false positive reactions arising from soluble matter included in the suspensions. Pre-immunization sera were included in the tests, providing a control against non-specific antibodies and (these being negative) also against false positive reactions to constituents of the gel, by either antigens or sera. Agar was not used in the preparation of the antigens.

RESULTS

Sera were prepared against *Haemophilus vaginalis* (Edmunds) strains GP1, GP2, GP3, GP6, GP7; *H. vaginalis* (Gardner & Dukes) G-D; *H. vaginalis* (Amies & Jones) H689 and Dingham; *Corynebacterium cervicis* strains 12EA, 9T, 26T. These 11 sera were tested against all 21 strains of bacteria listed in the previous section. The results are shown in Table 1. The vertical lines represent the number of distinguishable lines in each positive precipitin reaction. It appears that on this basis the

organisms formed three groups: one containing the *Haemophilus vaginalis* strains Edmunds and Gardner & Dukes; the second, the Haemophilus species of Amies & Jones and the NCTC strains; the third, *Corynebacterium cervicis*. A degree of cross-relationship existed between some members of different groups.

Table 1. *Gel-diffusion precipitin reactions*



——, negative; —||—, vertical strokes represent number of visible lines of precipitation; 0, not performed.

To identify further the antigens responsible for these positive reactions, each serum was tested simultaneously against all the strains with which it reacted. The antigens were placed in cups either around the circumference of the cup containing the serum, or parallel with a trough of serum. In this way the identity or otherwise of lines of precipitation could be determined. These analyses were repeated with different batches of antigens, prepared on at least three different occasions in each case. This was done because the strength of individual lines varied from preparation to preparation of antigen, both relatively and absolutely, probably because of the hazards of preparation by ultrasonic disintegration and possibly also because of antigenic variation. Some of the antigenic components represented in the sera

reacted more strongly with related than with homologous antigens. This phenomenon became more important when PPLO antigens were examined (see Pease, 1965).

The results of these analyses are given in Table 2. The antigens are numbered in arabic numerals, and their position in the series has no significance. Factors appearing most strongly as antibodies in the homologous bacteria are shown in parentheses.

Table 2. *Antigenic structure of some Haemophilus and Corynebacterium organisms*

Antigens are numbered arbitrarily. Those shown in parentheses were regularly demonstrable in sera but only in concentrated antigenic preparations.

Specific groups	Antigens
Haemophilus vaginalis	
(*a*) Edmunds strains	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, (11), (17)
(*b*) Gardner & Dukes strains	(1), 2, 3, 8, 9, (10)
H. influenzae	
(*a*) Respiratory strains	17, 18, 32, 33, 34, 35, 36, 38
(*b*) Amies & Jones strains	11, 17, 18, 31, 32, 33, 34, 35, 36, 37, 38
Corynebacterium cervicis strains	11, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24

DISCUSSION

There are numerous suggestions in the literature of a relationship between the genera *Haemophilus* and *Corynebacterium*. Deacon, Albritton, Edmundson & Olansky (1954) and McKay & Truscott (1959) described Gram-positive corynebacterium-like variants of *H. ducreyi* and *H. gallinarum*, respectively. The organism described as *H. vaginalis* by Gardner & Dukes (1955) and by Edmunds (1960, 1962) was considered by Lapage (1961) to resemble a corynebacterium, and was renamed *C. vaginale* by Zinnemann & Turner (1962, 1963). Pease & Bisset (1962) suggested that the genus *Haemophilus* was a Gram-negative derivative of the genus *Corynebacterium*.

The results of the present investigation confirm that the *Haemophilus vaginalis* of Amies & Jones (1957) is serologically very close to *H. influenzae*, and that both species have antigens in common with the corynebacterium-like *H. vaginalis* of Gardner & Dukes, and with *C. cervicis* (Laughton), both of which vary between a Gram-positive and a Gram-negative phase. The relationship between these genera, which have been especially closely associated with PPLO, is of great interest in the solution of the problem of the origin and relationships of PPLO. The antigens defined in the present work also appear to be of significance to the problem of the antigenic structure of PPLO and their presumed parent bacteria (Pease, 1965).

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The Antigenic Structure of PPLO (*Mycoplasma hominis*) and Related Bacteria

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SUMMARY

By gel-diffusion precipitin and antigen-absorption tests, 34 strains of PPLO (*Mycoplasma hominis*, types 1–4) were examined, and 15 antigenic components recognized. Antigens *a*, *b*, *f* and *g* were common to types 1, 2 and 4; *c* was specific to type 1; *h* was specific to type 2. Type 3 was antigenically unrelated to the remainder. Its two recognizable components were named *j* and *k*. Three further antigens in type 1 were recognized as 17, 39 and 40, previously defined in strains of Haemophilus and Corynebacterium associated with mycoplasma-like variants (Pease & Laughton, 1965); antigen 40 also occurred in type 2. Two diffusible antigens, α and β, were detected in strains of types 1 and 4; these and also *g* were found to be shared with the bacterial strains.

All genital strains of mycoplasma isolated in this laboratory belonged to type 1, all oral strains to type 4; one rat strain belonged to type 2.

Distribution of antigenic components in strains of mycoplasma and in different strains of bacteria, including those believed to be derived from mycoplasma (or vice versa), and also in others, notably *Haemophilus influenzae*, supports previous claims of relationship between *Mycoplasma hominis* types 1, 2 and 4, Corynebacterium and Haemophilus. The validity of Mycoplasma as a distinct group of micro-organisms is thus called into question.

INTRODUCTION

Several attempts have been made to classify *Mycoplasma hominis* and similar PPLO by serological methods, including: complement fixation (Huijsmans-Evers & Ruys, 1956; Card, 1959; Coriell, Fabrizio & Wilson, 1959; Taylor-Robinson, Somerson, Turner & Chanock, 1963); agglutination (Norman, Saslaw & Kuhn, 1950; Nicol & Edward, 1953; Edward & Fitzgerald, 1954; Tourtelotte & Jacobs, 1959); growth inhibition by antibody (Nicol & Edward, 1953; Edward & Fitzgerald, 1954; Huijsmans-Evers & Ruys, 1956); and gel precipitation (Villemot & Provost, 1959; Pease & Laughton, 1962; Pease, 1962; Taylor-Robinson *et al.* 1963; Lemcke, 1964). There is general agreement that human mycoplasmas fall into four main groups, represented by *M. hominis*, types 1–4 (Huijsmans-Evers & Ruys, 1956; Edward & Freundt, 1956). Complement-fixation and gel-diffusion precipitins have provided evidence of relationship between these groups, especially between types 1 and 4; the degree of relationship, the place of types 2 and 3 and of other PPLO especially those of animal origin, has remained obscure. Specific names have also been applied to the various types by some authorities.

The purpose of the present work was to determine the relationship between those PPLO claimed to be L-forms of Corynebacterium and Haemophilus species (Pease &

Laughton, 1962; Pease, 1962; Pease & Bisset, 1962), to determine the number and distribution of the antigens, and to investigate the possible antigenic relationships between PPLO and bacteria. The antigenic structure of some strains of bacteria believed to be derived from, or associated with, PPLO was described by Pease & Laughton (1965).

METHODS

Mycoplasma strains used. Fifteen strains (Gt. 1 to Gt. 15 inclusive) were isolated from human genital tracts by the methods of Pease & Laughton (1962). Eleven human oral strains (Or. 1 to Or. 11 inclusive) and one strain (E) from the respiratory passages of a rat were isolated directly upon serum agar by Mr B. C. Cole in this laboratory. Three strains of type 1 (H 26, D 419, H 50 R), one of type 2 (Campo), one of type 3 (G), and one of type 4 (H 110) were provided by Dr D. G. ff. Edward (Wellcome Research Laboratories).

Bacterial strains used. Seven strains of *Corynebacterium cervicis*, ten of *Haemophilus vaginalis*, five of *H. influenzae* and one of *H. parainfluenzae*, listed by Pease & Laughton (1965), were used. Two strains of Corynebacterium derived by Mr B. C. Cole from PPLO Or. 4 and Or. 9 (Bact. Or. 4 and Bact. Or. 9), one Corynebacterium (D 5) derived from mycoplasma (Campo) by Dr H. E. Morton, a strain of *C. cervicis* (SM) described by Pease (1962), associated with a *Streptobacillus moniliformis* strain. One strain each of *H. aegyptius* and *H. aphrophilus* from the National Collection of Type Cultures.

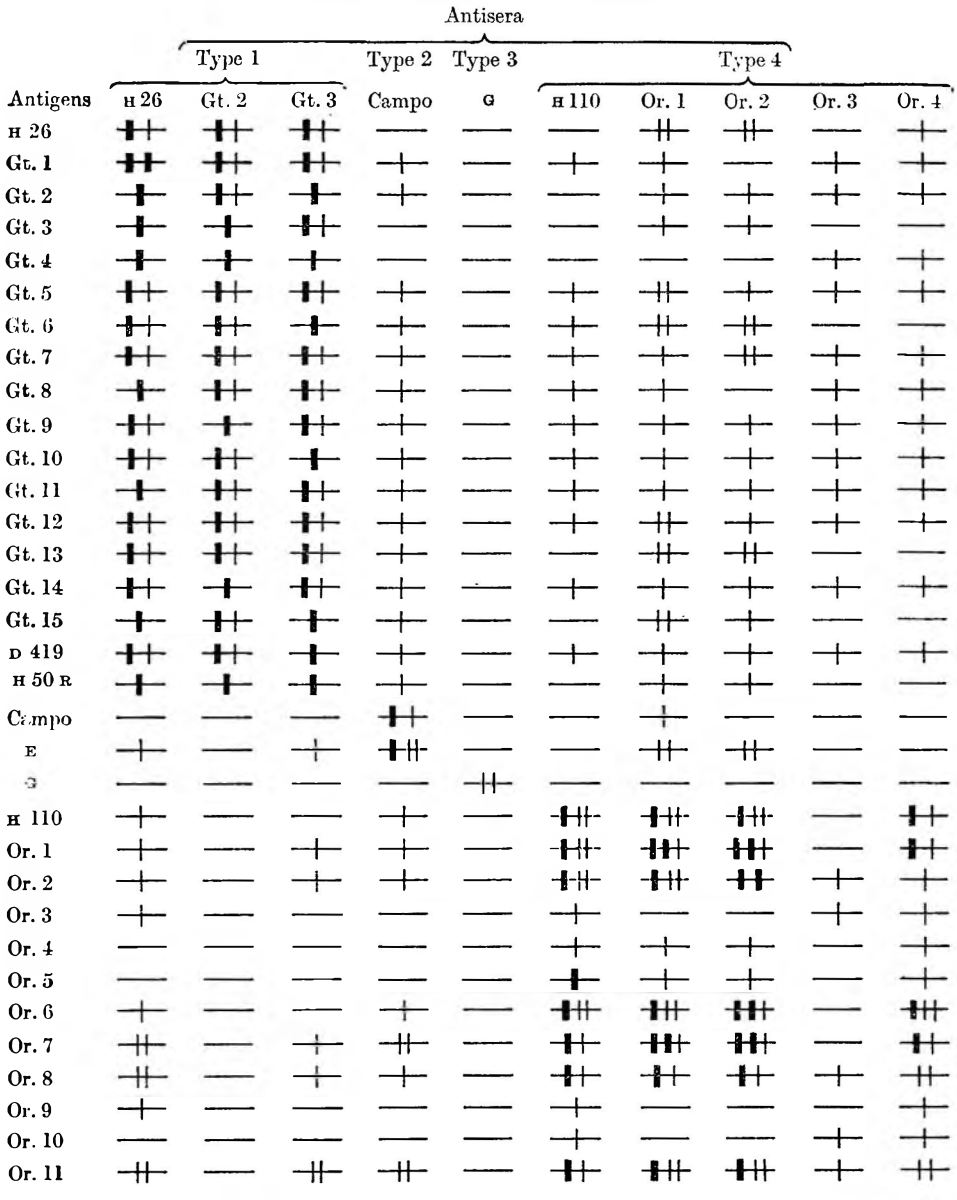
Serology. For the preparation of antigens, 2 l. liquid medium (composition: Lab Lemco 1%, w/v; Oxoid yeast extract 0·5%, w/v; Protease peptone 1·0%, w/v; NaCl 0·5%, w/v; horse serum 10%, v/v, with arginine 0·2%, w/v, for the oral strains; pH 7·8) were inoculated with the centrifuged deposit from a 48 hr culture in the same medium. The culture was incubated for 5 days at 37° and the organisms centrifuged down and washed 3 times in physiological saline containing 0·2% (v/v) of 40% (w/v) formaldehyde. The entire yield of organisms was again concentrated to 2 ml. in formol saline and subjected to ultrasonic disintegration for 5 min. Standardization by opacity was impracticable. The disintegrated organisms were centrifuged and the deposit was made up to 6 ml. in formol saline.

Immunization and gel-diffusion precipitin tests were done by the techniques described by Pease & Laughton (1965) and similar controls were used. It was also possible to grow the antigenically important derived bacteria, Bact. Or. 4 and Bact. Or. 9, on media without Fildes extract, thus providing an additional control against false reactions from this source. A technique of antigenic absorption was also used. It was found convenient to reverse the usual procedure and to absorb antigen with serum, instead of vice versa. One drop of serum added to a cup containing one drop of antigen was usually adequate to absorb all the antigens for which antibodies were present in the serum, as controlled by the same serum in a contiguous cup. For direct comparison with such absorbed antigens, the reactions were made at the same dilution (50%). This technique permitted absorptions to be done successfully with the very small quantities of mycoplasma antigens that were available.

RESULTS

Ten antisera were prepared against *Mycoplasma hominis* strains Gt. 2, Gt. 3; Or. 1, Or. 2, Or. 3, Or. 4; H 26, Campo, G, H 110. These antisera were tested against all 33 strains. The results are shown in Table 1, where the vertical lines represent the

Table 1. *Gel-diffusion precipitin reactions, showing relationships between strains of Mycoplasma hominis*



—, negative; ⊣⊢, defined lines of precipitation;

▮, thick, complex or multiple lines of precipitation.

number of distinguishable lines in each positive precipitin reaction. From Table 1 it is apparent that the genital strains formed a group with the *Mycoplasma hominis* type 1 strains from outside sources; this group may simply be referred to as type 1. The oral strains formed a separate group with the type 4 strain; this group may now be referred to as type 4. Between types 1 and 4 there were several cross-reactions. Type 2 is represented by strain Campo and one rat strain, but it has cross-reactions with types 1 and 4. Type 3 is represented by one strain G and showed no relationship to any other.

Cross-reactions between PPLO groups

The reactions between type 1 antigens and types 2 and 4 antisera showed only single lines in most cases, and never more than two lines. The identity of the corresponding antigens was established by coincidence of these lines, where the various antigenic preparations made from the members of the type 1 group were tested simultaneously against type 4 sera. The commonest type 2 or type 4 antigen shared by type 1 was thus defined and designated *a*; the second, less common, was designated *b*. (Table 2).

The phenomenon referred to by Pease & Laughton (1965), where antigens were more clearly definable by means of the homologous antibody than by the antigen, occurred in the present work also, and was of importance in the application of precipitin or antigen absorption to the further analysis of the antigenic structure of these groups. Type 4 antigens did not react, at normal concentrations, with Gt. 2 (type 1) antiserum, although Gt. 2 antigen reacted with all type 4 antisera, giving either one or two lines; but Gt. 2 antiserum, mixed with homologous antigen or with any other type 1 antigen, had the effect of eliminating these lines, i.e. of absorbing antigens *a* and *b*. Thus the presence of antigens *a* and *b* in type 4 strains, presumably in too small quantities to produce a visible line of precipitation, was indicated. This was verified by increasing the relative concentration of the type 4 antigens, as far as the technique allowed, when a faint reaction was produced.

Absorption of type 1 antigens containing components *a* and *b*, with any type 1 antiserum (irrespective of whether both *a* and *b* showed strongly in cross-reaction of type 4 antiserum with the type 1 antigen homologous with the absorbing antiserum), had the effect of removing antigens *a* and *b*. Thus it appears that antigens *a* and *b* were normally present in type 1 strains although only one or the other might appear as a visible line in a cross-reaction.

Since antigens *a* and *b* were common to types 1, 2 and 4, it was possible to determine whether any antigens other than these were present in the type 1 homologous system. This was done in two ways. Type 1 antigen (Gt. 6) with demonstrable components *a* and *b* was placed in the centre cup and surrounded by cups containing types 2 and 4 (Or. 1) sera, interspaced with type 1 antisera (Gt. 2 and 3 and H 26). Two lines, representing *a* and *b*, showed continuously around the central cup. Opposite the type 1 sera a further strong line appeared, overlapping the *a* and *b* lines where they deviated opposite the types 2 and 4 antisera. This further antigen, specific to type 1, was named *c*. It was also demonstrated by absorbing the type 1 (Gt. 6) antigen with type 4 (Or. 1) serum, which had the effect, in the same arrangement of sera and antigens as previously, of removing line *a* and *b*, and leaving line *c*. In a further test, with the absorbing antigen in the central cup, the type 1 antisera were placed in neighbouring cups. The residual line *c* was then continuous, showing

it to be present equally in strains Gt. 2, 3, H26. The identity of *c* in the remaining type 1 antigens, for which antisera were not available, was established by the continuity of lines when these antigens were placed in central cups and tested against the available antisera.

Table 2. *The antigenic structure of Mycoplasma hominis types*

Type 1	*a*, *b*, *c*, *f*, *g*
Type 2 (Campo)	*a*, *b*, *f*, *g*, *h*
Type 3, 'G'	*j*, *k*
Type 4	(*a*),* (*b*), *d*, *e*, *f*, *g*

* Letters in parentheses indicate factors normally present in sera but demonstrable only in concentrated antigenic preparations.

By the second of these methods, the existence in type 4 of antigens other than *a* and *b* was demonstrated. Each of two type 4 antigens (Or. 6 and Or. 7) was absorbed with each of two type 1 sera (Gt. 3 and Gt. 4). These four absorbed antigens were placed in central cups, each surrounded with cups containing five type 4 sera (Or. 1, Or. 2, Or. 3, Or. 4 and H110). Against these sera, the absorbed antigens gave two lines in each case, except with serum Or. 3, where one line only was discernible. The lines were continuous between neighbouring cups, and thus represent two further antigens, *d* and *e*, of which *d* only appears in Or. 3.

Two further antigens appearing in most type 4 strains were identified by their reaction against Gt. 3 and H26 sera, but against no other type 1 sera. These two antigens were distinguishable from *a*, *b* and *c* because they appeared only in reaction against these two sera, whereas all type 1 sera contained *a*, *b* and *c* antibodies in large amounts. They were distinguishable from *d* and *e* because they did not appear in, for example, Or. 4 and H110, where *d* and *e* were strongly represented. They were distinguishable from one another by absorption of Or. 8 with Gt. 3 or H26 serum, after which the antigen continued to react with the alternative serum. Each of these antigens formed a single line when the positive antigens were placed contiguously around the appropriate serum. These two antigens were named *f* and *g*. The type strain of type 2, Campo, by the same criteria, possessed antigens *a*, *b* and *f*, and also an antigen peculiar to the group, named *h*. The strain E, isolated from a rat, reacted very strongly with Campo serum, and possessed *a*, *f* and *g* in addition to *h*.

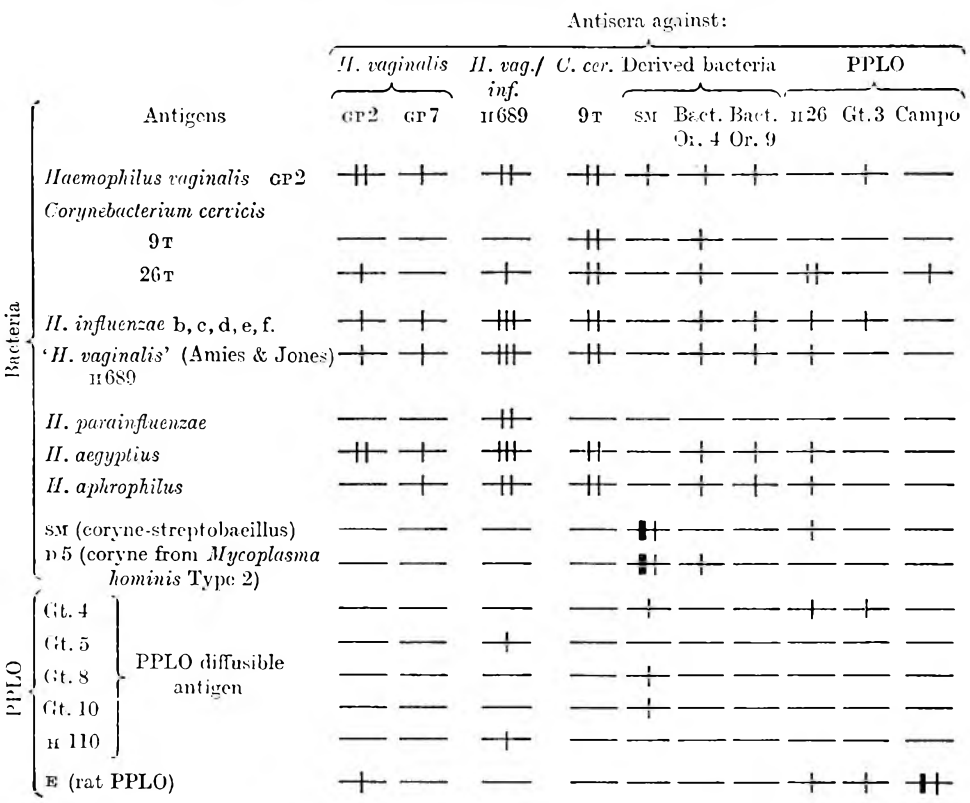
Type 3 was represented only by strain G, which showed two distinguishable lines with the homologous serum. These antigens are called *j* and *k* in Table 2.

Cross-reactions between PPLO and bacteria

Table 3 shows the cross-reactions between the bacterial and PPLO strains, using the sera listed above, those against the derived strains Bact. Or. 4 and Bact. Or. 9, and the corynebacterial variant of SM, and also those listed by Pease & Laughton (1965). The antigenic component 12, common to several bacterial strains (Table 4), was detected at this stage by the reaction of the sera Bact. Or. 4 and Bact. Or. 9 with both the Edmunds GP2 strain of *Haemophilus vaginalis* and the *H. influenzae* group. The line of precipitation proved to be continuous between contiguous, alternative cups containing the several types of antigen or antiserum. Relationships between bacterial strains isolated as such, and those derived from PPLO, were shown by

reactions of SM serum and antigen with D 5 antigen. By cross-absorption, two common antigens, 41 and 42, were identified. A third antigen was common to D 5, Bact. Or. 4 and 9T.

Table 3. *Cross-reactions between bacteria and PPLO*



The cross-relationships between PPLO and bacteria were examined further. The serum H 26 (*Mycoplasma hominis* type 1) reacted positively with most members of the *Haemophilus influenzae* group, as antigenically defined by Pease & Laughton (1965), an example being H 689 (*H. vaginalis*, Amies & Jones). When H 689 was placed opposite the sera H 26, Bact. Or. 4 and Bact. Or. 9, the line representing this precipitin reaction was continuous for all three. Thus the common antigen was present in these bacterial strains derived from PPLO. The identity of the antigen in reactions between H 26 (PPLO) serum and the remaining members of the *H. influenzae* group was established in the same way. This antigen, common to type 1 mycoplasma and to all listed strains of *H. influenzae*, was absorbed from *H. influenzae* f (non-capsulated) antigens by the following antibacterial sera: GP 2, GP 7 and 9T, but not by H 689. The antigen established in the scheme of Pease & Laughton (1965) which fulfils these requirements is 17, and it is thus concluded that 17 is an antigen common to mycoplasma, Haemophilus, Corynebacterium and also to the bacteria of corynebacterial morphology derived from mycoplasma.

The second common antigen was detectable by the reaction between Gt. 3 (mycoplasma type 1) antiserum and GP2 (*Haemophilus vaginalis*, Edmunds). This was distinct from the first common antigen (17) because there was no reaction between H26 or Bact. Or. 4 sera and GP2 antigen. By identity of lines, where GP2 antigen was

Table 4. *Distribution of antigens in PPLO and bacteria*

Specific types	Specific antigens	Group antigens
Haemophilus vaginalis	1, 2, 3, 4, 5, 6, 7, 8, 9, 10	11, 12, 17, 39
H. influenzae, *H. parainfluenzae*, '*H. vaginalis*' (A. & J.)	31, 32, 33, 34, 35, 36, 37, 38	11, 12, 17, 18, 39, α
Corynebacterium cervicis	15, 16, 19, 20, 21	11, 17, 18, 25, 40, *g*
Corynebacteria associated with Campo and with *Streptobacillus moniliformis*	41, 42	25, *g*, β
Corynebacteria associated with oral PPLO	43, 44, 45	12, 17, 25, 39, 40
Mycoplasma hominis type 1	*a*, *b*, *c*, *f*	17, 39, 40, *g*, α, β
M. hominis type 2	*a*, *b*, *f*, *h*	40, *g*
M. hominis type 4	*a*, *b*, *d*, *e*	*g*, α

surrounded by contiguous cups containing Gt. 3 serum, alternating with GP2 and Bact. Or. 9 sera, it was determined that the same antigen occurred in Bact. Or. 9. This antigen did not appear to have been previously determined and was named 39. A trace of antigen 39 appeared to exist in the rat strain E, and in *H. influenzae*.

The third and fourth common antigens were detectable by the reaction between H26 (mycoplasma type 1) serum and 26T (*Corynebacterium cervicis*; Laughton, 1954) antigen. Two lines were visible. These were distinguishable from antigen 17 because with H26 serum against 26T and *Haemophilus influenzae b* and *c* antigens, in contiguous cups, these lines of precipitation crossed, and thus showed non-identity with, the line between H26 and the *H. influenzae* strains. They were also distinct from 39 because 26T antigen did not react with Gt. 3 antiserum. One of these two lines was found, by the test of 26T antigen against antisera including H26, in contiguous cups, to be present in the Campo (*Mycoplasma hominis* type 2) and Bact. Or. 9 sera. The other showed lines of identity, in the same way, with type 1 and type 4 PPLO sera against 26T antigen, and by absorption was identified in H110, Or. 1, 2, 3, 4, 6, 8, 9 and 11; Gt. 2, 4, 6, 7, 10, 11, 15 and 16, and the streptobacillus SM (a trace). This antigen appears to be *g*; the second line in the 26T, H26 reaction was not identifiable with any previously named antigen and was called 40.

Diffusible antigens

Two diffusible antigens, of the type described by Freimer, Krause & McCarty (1959) and Pease (1963) were also identified, to a limited extent. The antigens extracted from H110 and Gt. 5 medium reacted positively with H689 (*Haemophilus influenzae*, 'vaginalis', Amies and Jones) serum; the same antigen was detected in *H. influenzae* b (non-capsulated), by identity of lines. Extracts Gt. 4, Gt. 8 and Gt. 10 reacted with SM serum. These diffusible antigens were named α and β, respectively.

DISCUSSION

The evidence in Tables 1 and 2 confirms the conclusions of Huijsmans-Evers & Ruys (1956), Edward & Freundt (1956) and subsequent authors, that *Mycoplasma hominis* type 1 consists of human genital strains, and type 4 of human oral strains. Type 2, represented by Campo, also of genital origin, has close relationships with strains from the respiratory passages of rats. This is true not only of strain E, but also of other rat isolates (Lemcke, 1964; Morton, H. E., personal communication). Types 1, 2 and 4 are antigenically closely related, as is already known, and the shared antigens have been defined in this paper. Type 3, of which very few strains have been recorded, is apparently unrelated.

The distribution of shared antigens shown in Table 4 provides evidence that the corynebacterial variants which have been claimed to be derived from PPLO (Minck, 1953; Wittler, Cary & Lindberg, 1956; Smith, Peoples & Morton, 1957; Pease & Laughton, 1962; Pease, 1962; H. E. Morton, personal communication) and the unrecorded examples quoted in this paper are, in fact, what they appear to be, since those examined in this study had several antigens in common, both with PPLO and other corynebacteria, and with members of the Haemophilus groups. And it is notable that the strains of bacteria most recently derived from PPLO (the oral derivatives in Table 4) possess the largest number of shared antigens. This provides support for the view that the PPLO are the L-forms of these and similar bacteria and, if this is true, then the name *Mycoplasma hominis* is invalid. Apart from the question of bacterial origin, *M. hominis* type 1 shares antigens with *Haemophilus influenzae*, including the diffusible antigen α, which may be regarded as of cell-wall origin (Freimer *et al.* 1959; Pease, 1963), but none with *M. hominis* type 3 that was detectable by the methods employed.

Haemophilus may conceivably be a Gram-negative (or weakly Gram-positive) derivative of Corynebacterium, as *Streptobacillus moniliformis* has already proved to be (Pease, 1962). The relationship of Corynebacterium, Haemophilus and PPLO, as a degenerative series, was suggested by Pease & Bisset (1962). The apparent relationship between PPLO and *Haemophilus influenzae* is borne out by the similarity of their DNA base ratios (Sueoka, 1961). This bacterium not only has a hitherto unexplained relationship with myxoviruses, but also produces L-forms which resemble morphologically the filamentous stages of these large viruses (Pease & Bisset, 1962). Its close connexion with PPLO parasitic upon mammalian mucous membranes is of theoretical interest.

The evidence that PPLO are L-forms of corynebacteria and related genera now includes repeated experimental transformations of one into the other, similarity of base ratios, and the sharing of several identifiable antigens.

No claim for the completeness of this list of antigens can be made, nor is it known to what extent the differences between the types, as now defined, may be due to reversible phase changes.

Some of these antigens showed signs of lability, and it is possible that the tendency to imbalance between homologous antigens and antisera (cf. Pease & Laughton, 1965) was due to their acting as haptens, so that their degree of reactivity may have depended upon the molecular associations in which they occurred at different times. A similar imbalance between antibodies and homologous antigens was observed by

Brown & Nunemaker (1942) in reactions between *Streptobacillus moniliformis* and rat PPLO. A further point arises from these authors' work. Pease (1962) showed an antigenic relationship between *S. moniliformis* and human mycoplasmas. In the present work and in that of Lemcke (1964) the PPLO of rat origin proved to be type 2, and in the present work a bacterium (SM) associated with a strain of *S. moniliformis* fell into the same group, whereas the human oral and genital strains isolated in this laboratory were types 4 and 1, respectively. Although the classical type 2 (Campo) is a human genital strain, it is not comparable to the type 1 strains in frequency from this source, and thus it appears that the streptobacillus–mycoplasma type 2 group normally occurs in animals. The origins of type 3 are still obscure, since it is apparently unrelated to the remainder.

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Salicylate Degradation Test for Differentiation of *Mycobacterium fortuitum* from Other Mycobacteria

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SUMMARY

Most isolates of *Mycobacterium fortuitum* decomposed salicylate and stained black the Sauton agar medium containing salicylate (0·5 or 1 mg./ml.); and all isolates capable of decomposing salicylate were identified as *M. fortuitum*. Other species, including rapidly growing mycobacteria, did not decompose salicylate. Most but not all of the salicylate-decomposing isolates showed also PAS degradation and a positive 3-day arylsulphatase test. The salicylate-decomposing isolates utilized nitrite as sole nitrogen source and about one-half of them grew on a nitrite medium within 7 days. These reactions, salicylate degradation, PAS degradation, utilization of nitrite, and 3-day arylsulphatase activity, are shown to be useful for identifying *M. fortuitum*. The 3-day arylsulphatase test showed some exceptional cases; therefore, a positive reaction in this test was not sufficient to identify *M. fortuitum*. Positive reactions in the above four tests seemed to mean that an isolate is certainly *M. fortuitum*.

INTRODUCTION

Lehmann (1947, 1961) stated that salicylate exhibited some bacteriostatic action against tubercle bacilli in Sauton medium. The present author (Tsukamura, 1962) examined the susceptibility to sodium salicylate of various mycobacterial strains in an egg medium and found that *Mycobacterium tuberculosis* and *M. bovis* were specifically susceptible to this compound. Further examinations were made of rapidly growing mycobacteria on Sauton agar, with the expectation that the susceptibility might increase in this defined medium. During this study, it was found that some mycobacteria made black a Sauton agar containing high concentrations of salicylate. It was also found that most isolates which decomposed salicylate in this way showed a positive 3-day arylsulphatase test (Wayne, 1961; Kubica & Rigdon, 1961) and utilized nitrite as sole nitrogen source (Tsukamura & Tsukamura, 1965). It was noticed that these reactions (salicylate degradation, 3-day arylsulphatase activity, nitrite as sole nitrogen source) usually appeared together in an isolate. The salicylate-decomposing mycobacteria consisted of *M. fortuitum* and of several strains of rapidly growing unclassified mycobacteria. An attempt was made to classify these unclassified mycobacteria according to the methods described by Gordon & Smith (1955), *Bergey's Manual* (1957), and Gordon

& Mihm (1959), and according to the amidase tests described by Boenicke (1962). The results showed that these mycobacteria belonged to *M. fortuitum*. Thus, the salicylate degradation may be specific for *M. fortuitum*.

METHODS

Isolates. The following isolates were used: *Mycobacterium fortuitum* (no. 605, 606, 607: R. E. Gordon); *M. smegmatis* (Denken, Kyushu, Jucho, Takeo); *M. phlei* (Denken, Kyushu); rapidly growing unclassified mycobacteria isolated from soil sources in this laboratory (14 uncoloured and 8 coloured isolates); rapidly growing unclassified mycobacteria isolated from human patients (named Yamamoto, Nishiwaki, Mimura); scotochromogens (Runyon's group II, Runyon, 1955: 16 isolates); nonphotochromogens (Runyon's group III: 18 isolates); *M. kansasi* (Forbes 84, Bostrum D-35, Nagai); *M. avium* (A 71, 3717, 4110, 4121, 11755, Flamingo, Kirchberg, Nagoya 59); *M. balnei* (B 913, B 916: Lausanne); *M. ranae* (L-17: Lausanne); *M. platypoecilus* (one isolate: A. J. Ross); *M. piscium* (one isolate: A. J. Ross); *M. marinum* (one isolate: A. J. Ross); three isolates from tropical fishes (sf-2, LT, SW: S. Sato). Most of these isolates were supplied by Dr K. Konno (Research Institute for Tuberculosis and Leprosy, Tohoku University), Professor K. Takeya (Department of Bacteriology, Kyushu University) and Professor S. Hibino (First Department of Internal Medicine, Nagoya University).

Salicylate degradation. This was observed in a modified Sauton medium agar containing sodium salicylate (0·5 or 1 mg./ml.). The composition of the Sauton agar was as follows: glycerol, 50 ml.; sodium glutamate, 4·0 g.; KH_2PO_4, 0·5 g.; $MgSO_4.7H_2O$, 0·5 g.; sodium citrate, 2·0 g.; ferric ammonium citrate, 0·05 g.; purified agar (Wako Pure Chemical Co., Osaka, Japan), 30 g.; distilled water, 950 ml. This was adjusted to pH 7·0 and sodium salicylate was added to the medium, before sterilization, to final concentrations 0, 0·5 or 1 mg./ml. The medium was poured in 8 ml. quantities into tubes (170 × 17 mm.) and sterilized by autoclaving at 115° for 30 min., sloped and cooled. The medium was then surface inoculated with one loopful of the stock cultures and incubated at 37°. Growth and change in colour were observed after 1 and 2 weeks of incubation.

Nitrite utilization as sole nitrogen source. This was tested on the slopes of the following medium: $NaNO_2$, 1·38 g. (0·02M); KH_2PO_4, 0·5 g.; $MgSO_4.7H_2O$, 0·5 g.; sodium citrate, 2·0 g.; glycerol, 50 ml.; purified agar, 30 g.; distilled water, 950 ml.; adjusted to pH 7·0 by addition of 10% (w/v) KOH. Sodium nitrite was sterilized by Seitz-filtration and added to the medium aseptically. The slopes were surface inoculated with one loopful of the test strain. Growth was observed every week until the end of the third week and recorded by comparison with the growth on control medium without added nitrogen source.

Three-day arylsulphatase test. This was done according to the method of Wayne (1961).

p-Aminosalicylate (PAS) degradation. Degradation of sodium *p*-aminosalicylate (PAS) was tested according to the method described by Tsukamura (1961*a*, *b*). It was observed as a blackening of Ogawa egg medium containing PAS 1 mg./ml. Observation was made after 1 week of incubation at 37°.

Amidase tests. These tests were done according to Boenicke (1962). Time of incubation was 15 hr. at 37°.

Utilization of organic acids as sole carbon sources. This was tested on the following medium: $(NH_4)_2SO_4$, 2·64 g.; KH_2PO_4, 0·5 g.; $MgSO_4.7H_2O$, 0·5 g.; purified agar, 30 g.; distilled water, 1000 ml.; adjusted to pH 7·0. Solutions of sodium salts, acetate, citrate, succinate, malate, pyruvate and benzoate, were sterilized separately by heating at 100° for 5 min. and added to the above medium aseptically to give final concentrations of 0·01 M. Growth was observed in comparison with control medium without added carbon source after 1 and 2 weeks of incubation at 37°.

Acid formation from carbohydrates. This was observed in the following medium: NH_4Cl, 2·0 g.; KH_2PO_4, 0·5 g.; $MgSO_4.7H_2O$, 0·5 g.; 0·2 % (w/v) bromthymol blue solution in 0·02 M-NaOH, 20 ml.; purified agar, 30 g.; distilled water, 1000 ml.; adjusted to pH 7·0. This medium was sterilized by autoclaving at 115° for 30 min. The carbohydrate solutions were sterilized separately by heating at 100° for 5 min. and added to medium aseptically to a final concentration of 0·5 % (w/v). The slopes of this medium were inoculated with one loopful of organisms, and acid formation examined after 1 and 2 weeks of incubation at 37°. The following carbohydrates were used: glucose, mannose, galactose, arabinose, xylose, rhamnose, trehalose, lactose, raffinose, inositol, mannitol, sorbitol.

Nitrate reduction. The bacteria to final concentration of 10 mg. wet weight bacteria/ml. were suspended in 5 ml. 0·067 M-phosphate buffer (pH 7·1) containing 0·1 % (w/v) $NaNO_3$. The suspension was incubated at 37° for 16 hr. and tested for nitrite. Nitrite was examined by addition of two drops of 2 % (w/v) *p*-dimethylaminobenzaldehyde in 10 % (v/v) HCl + 1 ml. 10 % (v/v) HCl. Control tubes contained heat-killed bacteria.

Salt tolerance. This was examined in glycerol broth containing 5 % (w/v) NaCl. Growth was observed after incubation for 2 weeks at 37°.

Temperature response. Growth was observed on Ogawa egg medium and Löwenstein–Jensen medium after 1 and 2 weeks of incubation at 28°, 37°, 45°, 52°.

RESULTS

The results of the salicylate degradation, PAS degradation, nitrite utilization and 3-day arylsulphatase test with various mycobacterial isolates are shown in Table 1. Among the already classified mycobacteria, only *Mycobacterium fortuitum* showed a positive reaction in two or more of these tests. Nitrite was used as nitrogen source by *M. fortuitum* and gave growth within 1 week. Nitrite was also used as sole nitrogen source by *M. smegmatis* and *M. phlei*, but visible growth of these two was seen only after incubation for 2–3 weeks.

Among forty, classified or unclassified, rapidly growing mycobacteria, twenty isolates showed one or more positive reactions in the four distinguishing tests. Among these isolates, all of the strains of *Mycobacterium smegmatis* and *M. phlei* tested showed only use of nitrite as sole nitrogen source, showing visible growth after incubation for 2 or 3 weeks; the other activities were negative with these two organisms. Thus, these two species were separated from other strains. Among these latter strains, which are listed in Table 2, excepting the strain no. 315 which showed only the positive 3-day arylsulphatase test, all strains including *M. fortuitum*

showed at least two positive reactions (Table 2). The amidase test (Boenicke, 1962), the utilization of organic acids as sole carbon sources, acid formation from carbohydrates, nitrate reduction and NaCl tolerance were tested on these rapidly growing mycobacteria; the results are listed in Table 2. The results showed that the

Table 1. *Salicylate degradation, p-aminosalicylate (PAS) degradation, nitrite utilization, and 3-day arylsulphatase activity by various mycobacteria*

Salicylate degradation and PAS degradation were observed after one week of incubation at 37°.

Mycobacterium strains	No. of strains tested	Salicylate degradation	PAS degradation	Nitrite utilization*	Three-day aryl-sulphatase activity
M. fortuitum	3	+(2)	+(2)	+(3)	+(3)
M. smegmatis	4	−	−	±(4)	−
M. phlei	2	−	−	±(2)	−
M. marinum	1	−	−	−	−
M. platypoecilus	1	−	−	−	−
M. ranae	1	−	−	−	−
M. balnei	2	−	−	−	−
M. piscium	1	−	−	−	−
M. avium	8	−	−	−	−
M. kansasi	3	−	−	−	−
Scotochromogen (Group II)	16	−	−	−	−
Nonphotochromogen (Group III)	18	−	−	±(5)	−
Rapid grower (Group IV)	3	+(2)	+(3)	+ or ±(3)	+(2)
Rapid grower (Soil sources)					
Uncoloured	14	+(7)	+(7)	+ or ±(7)	+(7)
Coloured	8	−	−	−	−

( ) indicates no. of strains showing the positive reaction.
*+, positive growth within one week; ±, positive growth within 3 weeks.

pattern of activity of unclassified, rapidly growing isolates from soil or human patients, excluding the strain no. 315, is similar to that found in the three reference strains of *M. fortuitum*.

DISCUSSION

Among the strains listed in Table 2, only the strain no. 315 differed markedly from others. This strain showed only a positive 3-day arylsulphatase test among the four distinguishing reactions. It showed no amidase activity, utilized only acetate among organic acids, and formed no acid from carbohydrates. It resembled *Mycobacterium aquae* (Boenicke, 1962) except that it was uncoloured. From this case, we learn that the positive 3-day arylsulphatase test is not always specific for *M. fortuitum*, even though it is sure this test is useful to screen *M. fortuitum*. On the contrary, the absence of this activity does not exclude the test strain from *M. fortuitum*. For example, strains no. 330 and Mimura showed no 3-day arylsulphatase activity, but were considered to be *M. fortuitum* from their over-all similarity in other characteristics (Table 2).

All strains listed in Table 2, including three strains already classified as *Myco-*

Table 2. *Physiological reactions of various strains of Mycobacterium in which one or more of the four distinguishing reactions were positive*

	Four distinguishing reactions				Amidase (A)					Organic acids as C sources (B)					Acid from carbohydrates (C)							
					1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
	Salicylate	*p*-amino = Salicylate	Nitrite used	3-day Arylsulphatase	Acetamide	Urea	Nicotinamide	Pyrazinamide	Allantoin	Acetate	Citrate	Succinate	Malate	Pyruvate	Glucose	Mannose	Trehalose	Inositol	Mannitol	Nitrate reduced	NaCl tolerated	Highest temperature for growth
M. fortuitum																						
No. 605	−	−	+	+	+	+	−	−	+	+	+	+	±	−	+	+	±	+*	+	+	+	37°
No. 606	+	+	+	+	+	+	−	+	−†	+	+	+	+	−	+	+	+	−	−	+	+	37°
No. 607	+	+	+	+	+	+	−	−	+	+	+	+	+	−	+	+	−*	−	−	+	+	37°
Rapid growers from soil (uncoloured)																						
No. 313	+	+	+	+	+	+	−	−	+	+	+	+	+	−	+	+	−*	−	−	+	+	37°
No. 330	+	+	+	−	+	+	+	+	+	+	+	+	+	−	+	+	+	−	+	+	−	37°
No. 302	+	+	±	+	+	+	−	−	+	−	+	+	+	−	+	+	+	−	−	+	±	37°
No. 306	+	+	±	+	−†	+	+	+	+	−	+	+	+	−	+	+	+	−	+	+	±	37°
No. 308	+	+	±	+	+	+	+	+	+	+	+	+	+	−	+	+	−*	−	+	+	+	37°
No. 334	+	+	±	+	+	+	+	+	+	−	+	+	+	−	+	+	+	−	−	+	+	37°
No. 335	+	+	+	+	+	+	+	+	+	+	+	+	−‡	+	+	+	+	−	−	+	+	37°
No. 315§	−	−	−	+	−†	−†	−	−	−†	+	−‡	−‡	−‡	−	−*	−*	−*	−	−	−	−	37°
Rapid growers from humans																						
Yamamoto	−	+	±	+	+	+	+	+	−†	−	−‡	+	+	−	+	+	+	−	−	−	+	37°
Nishiwaki	+	+	±	+	+	+	+	+	−†	+	+	+	+	−	+	+	+	−	−	−	+	37°
Mimura	+	+	+	−	−†	±	−	−	±	+	+	+	+	−	+	+	−*	−	+	+	+	37°

(A) All strains were negative with benzamide, isonicotinamide, salicylamide, succinamide, malonamide.
(B) All strains were negative with benzoate.
(C) All strains were negative with galactose, arabinose, xylose, rhamnose, lactose, raffinose, sorbitol.
* Deviation from the description of *Bergey's Manual* (1957) and Gordon & Smith (1955) for *Mycobacterium fortuitum*.
† Deviation from the description of *M. fortuitum* by Boenicke (1952).
‡ Deviation from the description of *M. fortuitum* by Gordon & Smith (1955) who tested on the utilization of citrate, succinate, malate, benzoate.
§ No. 315 showed pattern of *M. aquae*, except that it was uncoloured; no acid from carbohydrates.
Symbols for nitrite utilization: +, positive growth within one week; ±, positive growth within 2 weeks; −, negative growth after 3 weeks.

bacterium fortuitum, showed a few deviations from the typical characteristics of *M. fortuitum* (Gordon & Smith, 1955; *Bergey's Manual*, 1957; Gordon & Mihm, 1959), but the unclassified strains shown in Table 2, excluding strain no. 315, were thought to be *M. fortuitum* from their similarity to the reference strains. It was noted that these strains showed two or more positive distinguishing reactions (Table 2). On the other hand, no other strains showed such reactions (Table 1). In view of these results, it was thought that the four distinguishing reactions are useful to distinguish *M. fortuitum* from other mycobacteria.

Wayne (1961) and Kubica & Rigdon (1961) stated that *Mycobacterium fortuitum* may be differentiated from other rapidly growing mycobacteria by its positive arylsulphatase test. However, as shown in this study, there are some exceptional cases (strains no. 315, 330, Mimura). Growth at 7 days with nitrite as sole nitrogen source may be specific for *M. fortuitum* (Tsukamura & Tsukamura, 1965), but a negative reaction does not exclude *M. fortuitum* (Table 2). Similarly, positive reactions of the salicylate degradation and the PAS degradation seem to be specific for *M. fortuitum*; but all strains of *M. fortuitum* do not show these reactions (Table 2). We must say that it is impossible to detect *M. fortuitum* by one characteristic, but, from the results of this study, it seems to be possible to do so by use of four marker characteristics. When a test strain shows two or more positive reactions among the four distinguishing reactions (salicylate degradation, PAS degradation, growth on nitrite medium within 7 days, 3-day arylsulphatase test), the strain may be identified as *M. fortuitum*.

Cattaneo, Morellini, Penso & Vicari (1954) and Penso, Cattaneo, Morellini & Vicari (1955) stated that *Mycobacterium piscium* and a number of isolates from cold-blooded animals darkened PAS-containing media. It was a peculiar fact that they stated that *M. minetti* (*M. fortuitum*) showed a negative reaction, based upon the observation on one isolate. Tsukamura (1961 *a*, *b*) observed that certain mycobacteria isolated from the soil blackened PAS-containing media (at that time, the author did not know of the Italian work) and showed that the coloured formazan was due to the change of PAS catechol.

There remains some question as to whether *Mycobacterium fortuitum* is the only species degrading PAS, because the Italian authors stated that *M. piscium* and other mycobacteria from cold-blooded animals darkened the PAS medium. In the present study, it was observed that one strain of *M. piscium* and one strain (B 913) of *M. balnei* made a PAS medium brown after more than 2 weeks. Three strains from tropical fishes also made the medium dark after incubation for 2–3 weeks. However, it seemed easy to distinguish the reaction of *M. fortuitum* from the false reaction of other mycobacteria, since the *M. fortuitum* reaction was much stronger and appeared sooner, i.e. after 1 week incubation.

In contrast to the PAS degradation, salicylate degradation appeared to be clearly restricted to *M. fortuitum*. Growth of the mycobacteria, which showed a false reaction in the PAS degradation, was inhibited on the Sauton agar containing sodium salicylate 0·5 or 1 mg./ml., and no change in colour was observed. In addition, salicylate degradation differed from the PAS degradation. This was shown by the fact that the strain Yamamoto decomposed PAS but did not decompose salicylate (Table 2). The degradation product of salicylate by *M. fortuitum* has now been shown to be catechol (Tsukamura 1965).

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Conversion of Salicylate to Catechol by *Mycobacterium fortuitum*

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SUMMARY

It was reported in a previous paper that most strains of *Mycobacterium fortuitum* blackened a modified Sauton medium agar containing 0·1 % (w/v) sodium salicylate. The mechanism of this blackening has been investigated. When a modified Sauton medium containing 0·1 % salicylate was inoculated with any of three strains of *M. fortuitum*, the medium became black after incubation for four days at 37°. The brownish black formazan was isolated by paper chromatography and compared with formazans from the same medium containing 0·1 % (w/v) catechol inoculated with the test organisms or without inoculation. These formazans showed similar R_F values in three solvent systems and the same absorption spectra (maximum absorption at below 220 and 275 mμ). It has been shown that *M. fortuitum* is capable of converting salicylate to catechol and that the formazan in the medium is an oxidation product of catechol.

INTRODUCTION

The author (Tsukamura, 1965) had found that *Mycobacterium fortuitum* decomposed salicylate and blackened a modified Sauton medium agar containing salicylate and that this reaction was specific for *M. fortuitum*. The present work is concerned with the mechanism of the blackening by *M. fortuitum* of medium containing salicylate.

METHODS

Strains. Mycobacterium fortuitum, nos. 606, 607 and 302, were used. As described previously, these strains caused a marked blackening of a modified Sauton medium agar containing 0·1 % (w/v) sodium salicylate within 7 days when the medium was inoculated with one loopful of the stock cultures of the test strains (Tsukamura, 1965).

Medium. A modified liquid Sauton medium was used, with the following composition: sodium glutamate, 4·0 g.; glycerol, 50 ml.; K_2HPO_4, 0·5 g.; $MgSO_4.7H_2O$, 0·5 g.; citric acid, 2·0 g.; ferric ammonium citrate, 0·05 g.; distilled water, 950 ml. The medium was adjusted to pH 7·0 by addition of 10 % (w/v) KOH, poured in 50 ml. quantities into 200 ml. Erlenmeyer flasks, and sterilized by autoclaving at 115° for 30 min. Sodium salicylate solution was sterilized by heating at 100° for 5 min. and added to the medium aseptically. Catechol was sterilized by Seitz filtration and added to medium aseptically. All reagents used were

extra pure grade (Katayama Chemical Co., Osaka, Japan). Sauton medium with 0·1 % (w/v) sodium salicylate and with 0·1 % (w/v) catechol were prepared.

Paper chromatography. Paper chromatography was done by upward flow. Toyo filter paper no. 50 was used. Three solvent systems were used: (1) *n*-butanol saturated with ammonia water (28 % NH_4OH); (2) *n*-butanol saturated with distilled water; (3) *n*-butanol + glacial acetic acid (4 + 1).

Absorption spectra were measured with a Hitachi spectrophotometer type EPU–2A (Hitachi Co., Tokyo, Japan).

RESULTS

The following series of media were set up: (1) uninoculated Sauton medium containing 0·1% sodium salicylate; (2) Sauton medium containing 0·1 % sodium salicylate inoculated with 300–400 mg. (moist weight) of test organism; (3) uninoculated Sauton medium containing 0·1 % catechol; (4) Sauton medium containing 0·1 % catechol inoculated with 300–400 mg. (moist weight) of test organism. All media were incubated at 37°.

Table 1. *R_F values in paper chromatograms of coloured formazans produced by Mycobacterium fortuitum nos. 606, 607, 302 when incubated with salicylate or catechol in Sauton medium*

		R_F values		
Solvent	Strain no.	Salicylate + organisms	Catechol + organisms	Catechol
A*	606	0·09	0·10	0·13
	607	0·10	0·13	0·13
	302	0·10	0·12	0·13
B	606	0·83	0·84	0·83
	607	0·83	0·82	0·83
	302	0·83	0·85	0·83
C	606	0·85	0·87	0·86
	607	0·84	0·86	0·86
	302	0·89	0·86	0·86

* Solvent A: *n*-butanol saturated with ammonia water. Solvent B: *n*-butanol saturated with distilled water. Solvent C: *n*-butanol + glacial acetic acid (4 + 1), by vol.
All samples showed another black spot at point 0 (R_F 0·00).

The two media with catechol showed an immediate violet colouring before inoculation. When each component of the medium was mixed with catechol, at the same final concentration, it was found that ferric ammonium citrate alone of the ingredients produced this colour. Catechol medium became dark brown or black after incubation for 4 days at 37°, even uninoculated medium in the absence of the test organisms. After incubation for 4 days medium (2), the inoculated Sauton media with salicylate, showed similar blackening. Only medium (1) uninoculated Sauton medium with 0·1% salicylate remained colourless.

Media (2) and (4) were centrifuged at 3000 rev./min. to remove the bacteria, and the supernatant fluids obtained from these with medium (3) were concentrated to $\frac{1}{2}$ to $\frac{1}{3}$ volume under reduced pressure. Evaporation was done for 2 days, during

which the media were maintained cold in the dark, the cold state being produced by the reduced pressure. The three concentrates (salicylate + organisms, catechol + organisms and catechol) were used for paper chromatography.

R_F values of the brownish black formazans in the concentrates are shown in Table 1. The formazans showed one spot in different solvent systems and their R_F values were almost identical. A brownish black spot remained at the original point. Elution of this spot with distilled water showed no marked absorption between 230 and 550 mμ, but showed an absorption in a region of less than 230 mμ; the nature of this spot is still unknown.

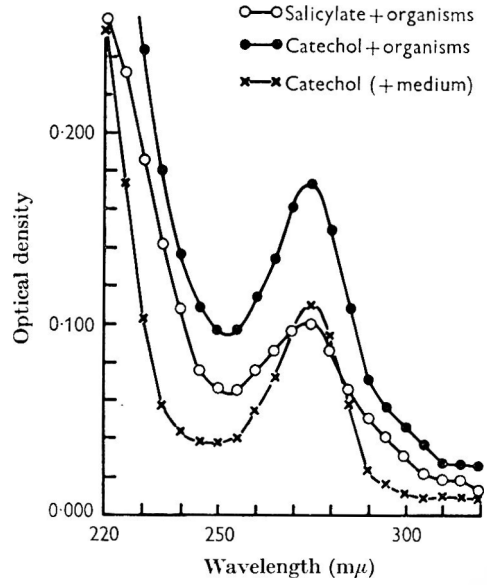
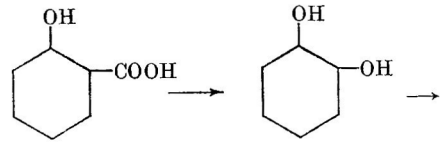


Fig. 1. Absorption spectra of the colour formazans produced by 'salicylate + organisms', 'catechol + organisms' and 'catechol'. In this example *M. fortuitum* no. 607 was used. Coloured sections of the paper chromatograms with R_F values 0·32–0·85 (Solvent: *n*-butanol saturated with distilled water) were eluted with distilled water and the absorption spectra of the eluates measured.

Coloured sections in the paper chromatograms developed in *n*-butanol saturated with water were cut out and extracted with distilled water for 16 hr. The extracts were examined by spectrophotometry. An example of the absorption spectra of the extracts is shown in Fig. 1. Extracts from the samples 'salicylate + organisms', 'catechol + organisms', and 'catechol' showed the same type of absorption spectra. Maximum absorptions were observed at a region of wavelength less than 220 mμ and at 275 mμ. Sodium salicylate showed maximum absorption at 230 and 295 mμ (data not shown).

In view of these results, the coloured formazan from salicylate was identified with the formazan from catechol since both formazans showed similar R_F values and similar absorption spectra. It was assumed that the salicylate was converted to catechol by *Mycobacterium fortuitum*, for when, instead of the living bacteria, heat-killed bacteria (100° for 5 min.) were added to salicylate medium, no blackening of the medium was observed.

It seems probable that the conversion of salicylate to catechol is enzymic. The reaction is described as follows:



Salicylic acid Catechol

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Thymine Incorporation and Metabolism by Various Classes of Thymine-less Bacteria

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SUMMARY

Escherichia coli strain 15T⁻ yielded 10^9 organisms from 1 μg. thymine and growth was continuous, not diphasic. Aminopterin-selected thymine-less bacteria, on the other hand, required 20–25 μg. thymine/ml. to sustain growth, although thymine incorporation was the same as for strain 15T⁻, approximately 10^{-9} μg./bacterium. In dearth of thymine the aminopterin-derived auxotrophs underwent thymine-less death (like strain 15T⁻), and in 5 μg. thymine/ml., after some initial growth, also underwent thymine-less death, although thymine uptake and DNA synthesis continued at a low rate. In 20 μg. thymine/ml. (and above) growth was diphasic. From these auxotrophs were derived, presumably the result of a second mutation, strains similar to strain 15T⁻. These differed from strain 15T⁻, however, as follows: they required 2 μg. thymine/ml. to initiate growth, they formed less thymidine phosphorylase, and they did not yield a particular class of revertant characteristic of strain 15T⁻. The aminopterin-derived auxotrophs were a thousand times more sensitive to inhibition by cytidine and uridine than were their double mutants or strain 15T⁻. This was the only trait discovered to correlate with their high thymine requirement. The high thymine requirement appeared not to be due to a permeability defect. Several classes of revertants were obtained from thymine-less bacteria. The majority regained thymidylate synthetase simultaneously with loss of ability to incorporate thymine efficiently. One class from strain 15T⁻ was unique: it regained thymidylate synthetase without losing thymine incorporation, and represented the one exception to the rule of mutual exclusion between these two traits. The aminopterin-derived auxotrophs were a distinctive, stable, and remarkably uniform class of thymine-less bacteria. They emphasized the uniqueness of strain 15T⁻, and illustrated the dual differentiation from a wild type possessed by their double mutants and also by strain 15T⁻.

INTRODUCTION

Numerous thymine-less auxotrophs of *Aerobacter aerogenes* have been isolated by the aminopterin method. All had a high thymine requirement compared with *Escherichia coli* strain 15T⁻. Likewise, aminopterin-selected thymine-less mutants from *E. coli* strain 15, from several *E. coli* K-12 strains, and from other bacteria yielded, without exception, auxotrophs with a high thymine requirement. All of Dr T. Okada's aminopterin-derived isolates (personal communication) required twenty times the concentration of thymine as did strain 15T⁻. Moreover, the thymine-less *Bacillus megaterium* isolated by Wachsman, Kemp & Hogg (1964) had a high thymine requirement. It would thus appear that thymine-less bacteria newly

isolated by the aminopterin method are different metabolically from *E. coli* 15T⁻. On the other hand, some aminopterin-derived mutants of *E. coli* reported in the literature show a low thymine requirement, more nearly that characteristic of strain 15T⁻. However, one of these, strain 15H⁻T⁻, was derived by Dr T. Okada (personal communication) as secondary growth from a high-thymine-requiring auxotroph. In the present study low thymine requirers routinely have been derived from high thymine requirers, presumably as a result of a second mutation. The purpose of the present report is to describe the relationship between various classes of thymineless bacteria, and to compare their thymine uptake and metabolism.

METHODS

Bacteria. Strains and pertinent information are listed in Table 1. *Escherichia coli* strains 15 and 15T⁻ were supplied by Dr Seymour S. Cohen. The latter organism is a u.v.-induced mutant, and was described by Barner & Cohen (1954). Two strains

Table 1. *Isolation and thymine requirement of bacterial strains*

Species	Strain designation	Parent	Method of isolation	Thymine necessary to sustain full growth (μg.)	Exogenous thymine incorporated/cell (μg.)
Aerobacter aerogenes NCTC 418	t	—	—	None (prototroph)	—
	t23	t	Aminopterin	25	—
	t23A	t23	Concentration gradient agar	1	—
	t23B	t23	Concentration gradient agar	~ 2	—
	t23-1	t23B	Heavy seeding on thymineless agar	None (prototroph, revertant)	—
	t23B-7	t23B	Heavy seeding on thymineless agar	1	—
Escherichia coli	15	—	—	None (prototroph)	$0{\cdot}05 \times 10^{-9}$ when grown in 5 μg. thymine/ml.
	15-1	15	Aminopterin	25	$0{\cdot}9 \times 10^{-9}$
	15-1B	15-1	Concentration gradient agar	~ 2	$0{\cdot}9 \times 10^{-9}$
	15-1-1	15-1	Heavy seeding on thymineless agar	None (prototroph, revertant)	—
	15T⁻	15	u.v.-induced	1	$0{\cdot}9 \times 10^{-9}$
	15T⁻-3	15T⁻	Heavy seeding on thymineless agar	None (prototroph, revertant)	$0{\cdot}9 \times 10^{-9}$ when grown in thymine
	15T⁻-6	15T⁻	Heavy seeding on thymineless agar	None (prototroph, revertant)	$0{\cdot}07 \times 10^{-9}$ when grown in 5 μg. thymine/ml.

are not listed: *E. coli* strain CT⁻ was supplied by Dr R. L. Sinsheimer; it was isolated by Dr P. C. Hanawalt by the aminopterin method following u.v. irradiation; *E. coli* strain 15H⁻T⁻ was received from Dr T. Okada.

Media. Buffer (pH 7·1) contained 0·6% (w/v) $Na_2HPO_4.7H_2O$ and 0·15% $NaH_2PO_4.H_2O$. Liquid minimal medium comprised in addition to buffer 0·10 %

NH_4Cl, 0·05 % KCl, 0·02 % $MgCl_2.5H_2O$, 0·03 % Na_2SO_4, and 0·3 % glucose (yield-limiting to 4×10^9 cells/ml.). Glucose, buffer, and the salt mixture (suitably concentrated) were autoclaved separately, were cooled, and were mixed aseptically. Stock cultures were maintained on minimal medium containing 1·6 % agar. For thymine-less bacteria 50 μg. thymidine/ml. was included. Minimal agar for plating contained 0·5 % glucose. Sterile buffer was used as diluent. For routine plate counts enriched agar was used. It contained 0·20 % K_2HPO_4, 0·2 % yeast extract (Difco), 0·5 % trypticase (Baltimore Biological Laboratory), 0·5 % glucose, 50 μg. thymidine/ml., and 1·6 % agar. The diluent had the same composition but without thymidine and agar.

Isolation of mutants. Thymine-less auxotrophs were acquired by two or more serial passages in liquid minimal medium supplemented with 200 μg. thymidine/ml. and 200 μg. aminopterin/ml. (Okada, Homma & Sonohara, 1962), followed by replicate plating (Lederberg & Lederberg, 1952) from enriched agar to minimal agar. Increasing the aminopterin concentration to 500 μg./ml. was necessary in some instances; incorporation of 200 μg. 5-bromo-deoxyuridine/ml. had no effect. Thymine-less bacteria obtained in this way required 20–25 μg. thymine/ml. in liquid minimal medium to sustain maximum growth. Strains with a low thymine requirement were isolated from these auxotrophs as follows: 10^9 washed cells in buffer were spread onto minimal agar, a cylinder of agar was cut from the middle of the plate with a cork borer 1 cm. in diameter, and 1 mg. thymine in 0·1 ml. water was placed therein. After 2 days of incubation satellite colonies appeared at the periphery of the confluent growth surrounding the depression. These colonies were streaked on enriched agar to ensure purity, and stock cultures were prepared on minimal agar containing thymine. The low thymine requirers will be referred to as double mutants. They may also be acquired by heavy seeding on minimal agar containing 2 μg. thymine/ml.

Chemicals. Leucovorin was a gift from Dr E. H. Dearborn, Lederle Laboratories. Aminopterin, folic acid, 5-bromo-deoxyuridine, the various deoxyribonucleotides, deoxyribonucleosides, and related compounds were purchased from California Corporation for Biochemical Research. Dihydrofolic acid, for use in dihydrofolic reductase assays, was prepared by $Na_2S_2O_4$ reduction of folic acid as described by Futterman (1957). Tetrahydrofolic acid, for thymidylate synthetase assays, was prepared by catalytic reduction of folic acid as described by Kisliuk (1957). Tritiated thymine and thymidine were purchased from Nuclear-Chicago Corporation.

Growth studies. Bacteria from a refrigerated stock culture were transferred to 5 ml. liquid minimal medium (supplemented as required) and incubated overnight without forced aeration. This fresh culture (washed twice with 20 ml. buffer at the centrifuge) served as inoculum for 10 ml. liquid medium in nephelometer (Coleman Model 9 Nepho-colorimeter) cuvettes with glass tubes through aluminium caps to permit gentle aeration with a Marco air pump. The cultures were incubated at 38° in a constant-temperature water bath. Growth was monitored by the following means: nephelometrically (against Coleman Nephelos standards) to measure mass, microscopically (Petroff–Hauser counting chamber) to assay for total organisms, and by means of plating (spreading method) to assay for viable organisms. Enriched agar served for routine plating. Where larger culture volumes were required, as in DNA assays, cultivation was in 130 ml. medium within gas-washing bottles and

aeration through the spargers was provided by compressed air. DNA assays were done on trichloroacetic acid extracts by the Burton (1956) modification of the Dische reaction.

Thymine incorporation. Minimal medium in the cuvettes was supplemented with tritiated thymine. After various periods of incubation 0·1 ml. samples were transferred to 0·9 ml. of 1 % aqueous formalin. Samples from cultures of high cell density were further diluted with formalin to maintain a cell concentration of 10^7/ml. Micropipettes were used to spread 0·05 ml. evenly onto degreased stainless-steel planchets. Thus the number of organisms on the planchets was held to approximately 10^6, and decrease in radioactivity due to self-absorption (by exponentially growing cells) was held to 25 %. The planchets were dried under an infrared lamp, exposed for 20 min. to fixative (95 % ethanol + glacial acetic acid, 3 + 1; van Tubergen & Setlow, 1961), passed through a series of ethanol solutions of decreasing concentration (from 95 to 25 %), and finally passed through two changes of water. In this procedure the cells (containing incorporated tritiated thymine) adhered to the planchets, whilst residual tritiated thymine was removed. The radioactivity was measured with a Packard gas-flow proportional counter. From the specific radioactivity of the thymine the amount of thymine incorporation by the organisms was calculated, and is plotted in the figures herein as μg. thymine incorporated/ml. culture.

Enzyme assays. Cell homogenates for dihydrofolic reductase and thymidylate synthetase assays were prepared as follows: 130 ml. of exponentially growing culture were harvested when the nephelometric measurement became 1000 (equivalent to 8×10^8 cells/ml.). The organisms were chilled, washed with cold buffer, suspended in 5 ml. of buffer, and homogenized with a French pressure cell (American Instrument Co.). The homogenates were centrifuged to remove particulate matter, and were assayed immediately and after overnight storage at $-22°$. The method described by Misra *et al.* (1961) and Mathews, Scrimgeour & Huennekins (1963) was employed for the dihydrofolic reductase assays, and the spectrophotometric method described by Friedkin (1963) was used for the thymidylate synthetase assays. Temperature was 23°. From the initial slopes of the absorbance *vs.* time curves the mμmoles substrate converted/min. were calculated. These results were then related to mg. homogenate protein. Homogenate protein was determined by the Folin–Ciocalteau method as described by Layne (1957).

It was desired to carry out the thymidine phosphorylase assays on organisms grown on thymine (5 μg./ml.) and on thymidine (50 μg./ml.). Since the activity of this enzyme decreases greatly during late exponential growth, the cultures were harvested after only three mass doublings, when the nephelometric measurement had become 200 (equivalent to 2×10^8 cells/ml.; 1×10^8 cells/ml. in the case of strain 15-1). Organisms from 700 ml. of culture were washed with cold 0·03 M-sodium phosphate buffer (pH 7·1), suspended in 5 ml. buffer, and homogenized with the French pressure cell. The homogenates were dialysed for 9–12 hr at 5° against demineralized water flowing at a rate of 1 l./hr, centrifuged to remove particulate matter, and stored at $-22°$. The homogenate was assayed as follows: 0·05 ml. was added to test tubes held at 0° and containing 1·2 μmole (3·0 μc.) tritiated thymidine in 0·65 ml. 0·03 M-sodium phosphate buffer (pH 7·1). The tubes were placed simultaneously in a 38° constant-temperature bath. At various intervals from 0 to 30 min. a tube was removed, the reaction terminated by the addition of 7 ml.

ethanol, and the resulting protein precipitate removed as described by McNutt (1955) for nucleoside transdeoxyribosidase assay. (The control received thymidine after the addition of ethanol.) The preparation was then taken to dryness at 50° under an air jet. After addition of carrier thymine and thymidine the dried material was chromatographed (descending) on Whatman no. 1 paper with water, requiring 4–5 hr. The u.v.-absorbing thymine and thymidine spots were eluted with 10 ml. of water. To detect breakdown of thymidine to thymine, radioactivity was measured on a 0·1 ml. sample added to 5 ml. of scintillation mixture [175 g. naphthalene, 7 g. 2,5-diphenyloxazole, and 0·375 g. 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene in 1 l. 1,4-dioxane] with a Packard liquid-scintillation spectrometer.

The same homogenates were used for nucleoside transdeoxyribosidase assays. Temperature was 38°. The method was that described by McNutt (1955) except that radioactivity rather than microbiological assay was used to measure the quantity of product in the eluted spots. The amount of thymidine formed was measured from water chromatograms as in the thymidine phosphorylase assays, and the fate of the reactants was analysed also from chromatograms developed with water-saturated butanol and with isobutyric acid + water + ammonia (198 ml. isobutyric acid + 100 ml. water + 4·5 ml. NH_4OH mixture).

RESULTS

Growth and thymine incorporation. The thymine deficiency of the aminopterin mutants was alleviated only by thymidylate, thymidine, and thymine. Neither thymidine triphosphate nor any of the other deoxyribonucleotides, deoxyribonucleosides, ribonucleotides, ribonucleosides, or free bases will replace these compounds. Nor will folic acid, leucovorin, riboflavin, B_{12}, or methionine, added singly or in combination.

Figure 1 illustrates the high thymine requirement of a typical aminopterin-selected auxotroph. Approximately 25 μg. thymine/ml. was necessary to sustain maximum growth, and most of the thymine remained in the culture filtrate. At low thymine concentrations, for instance 5 μg./ml., the culture increased somewhat in cell number, then underwent thymine-less death, and most of the thymine remained in the filtrate. This is in contrast to strain 15T$^-$ which in 1 μg. thymine/ml. showed a normal growth curve and yielded 10^9 organisms/ml. Aminopterin-derived auxotrophs such as strains t23 and 15-1 rapidly converted thymidine to thymine; diphasic growth by thymidine cultures was a manifestation of growth actually in thymine. Thymidylate was the best source of thymine for the aminopterin-derived auxotrophs; growth was not diphasic, and the cell yield was usually as good as with strain 15T$^-$.

Figure 2 illustrates thymine-less death and the lack of DNA synthesis by strain 15-1 in the absence of thymine. *A. aerogenes* strain t23 responded likewise. No thymidylate synthetase was detected with either mutant (Table 2). Moreover, Dr K. G. Lark (personal communication) cultivated both bacteria in D_2O with thymidylate, then transferred them to H_2O lacking thymidylate for 1 hr, and observed only one band from extracted DNA in the analytical centrifuge. Apparently little if any DNA was synthesized.

Although thymine-less death by strain 15-1 occurred in 5 μg. thymine/ml., DNA synthesis took place. Observe in Fig. 3 that the rates of DNA synthesis and thymine

incorporation became identical, but were less than the rate of mass increase; that the *initial* thymine uptake by strain 15-1 and 15T⁻ was the same; and that 15-1 approximately doubled in cell number before thymine-less death ensued. These observations lead to the idea that the poor growth of strain 15-1 in low thymine was not due to defective permeability. Strain 15-1 responded the same in 5 μg. thymine/ml. whether the inoculum was thymine-grown (30 μg./ml.) or thymidylate-grown (6 μg./ml.) Decreasing the temperature from 38° to 30° did not improve growth, but

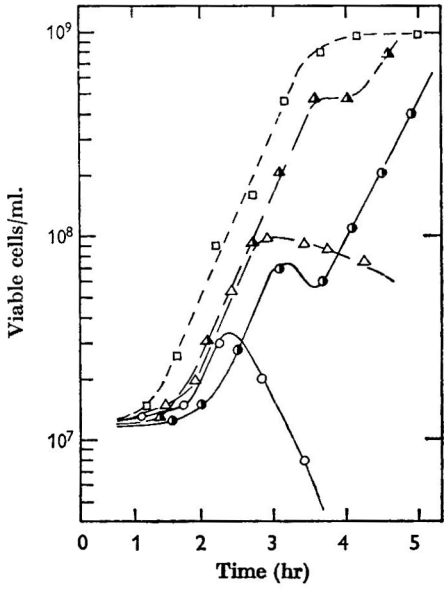

Fig. 1

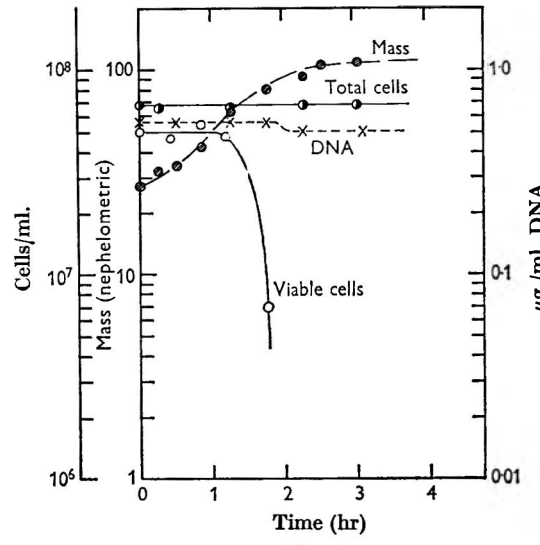

Fig. 2

Fig. 1. Growth of *Aerobacter aerogenes* strain t23 in liquid minimal medium. ○———, 0·04 μmole (5 μg.) thymine/ml.; ◑———, 0·2 μmole (25 μg.) thymine/ml.; △—— — ——, 0·04 μmole (9·7 μg.) thymidine/ml.; ▲—— ———, 0·2 μmole (48 μg.) thymidine/ml.; □ — — —, 0·008 μmole (2·7 μg.) thymidylic acid/ml.

Fig. 2. Response of *Escherichia coli* strain 15-1 in liquid minimal medium lacking thymine.

did delay thymine-less death for several hours. The rate of thymine incorporation by strain 15-1 (Fig. 3) was the same as by the parental strain 15 and by revertant strain 15T⁻-6. In these three instances the rate of thymine incorporation was much less than the rate of growth Contrariwise, strains 15T⁻, 15-1B, and 15T⁻-3 incorporated thymine at a rate equal to the growth rate (Fig. 4).

Figure 5 illustrates the response of strain 15-1 in 30 μg. thymine/ml. Note that the thymine uptake plateau preceded a doubled uptake, reflected by diphasic growth and break in DNA synthesis. Strain 15T⁻ also showed diphasic growth in high thymine concentrations, but to a much smaller degree.

In thymidine the double mutants and strain 15T⁻ grew equally well. This is in contrast to the poor thymidine incorporation by the parental strains (15-1 and t23) already cited, and led to an investigation of the enzymes involved in thymidine metabolism.

Thymidine phosphorylase. Strains 15 and 15T⁻ were cultivated in minimal medium containing 8 mμmoles/ml. (0·25 μc./ml.) tritiated thymidine at an initial

cell density of 5×10^7/ml. After the mass had increased five-fold, requiring 2 hr, strain 15 had converted 80% of the thymidine to thymine and strain 15T$^-$ had converted 85%. Strains 15-1 and 15T$^-$ (10^8 organisms/ml. buffer containing 28 mμmole/ml. tritiated thymidine) converted/ml., respectively, 6·2 mμmole and 5·4 mμmole thymidine to thymine after 5 min., and 19 mμmole and 17 mμmole thymidine to thymine after 30 min. Although there was apparently no relationship between thymidine phosphorylase activity and thymidine incorporation, the study

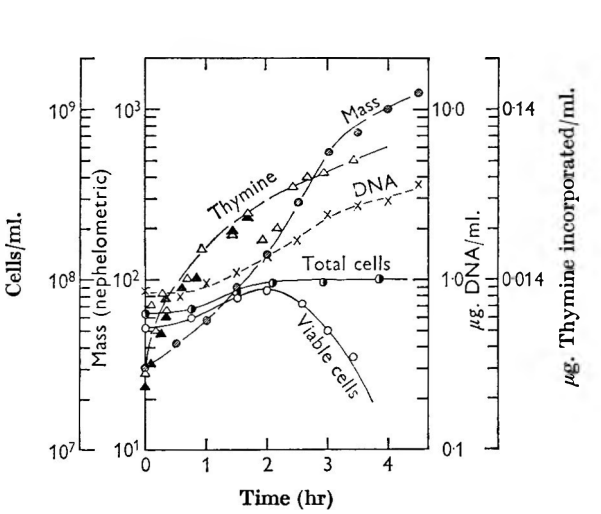

Fig. 3

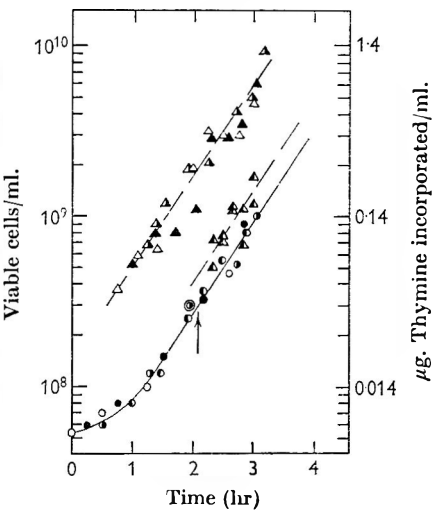

Fig. 4

Fig. 3. Response of *Escherichia coli* strain 15-1 in liquid minimal medium containing 5 μg. (4·4 μc.) tritiated thymine/ml. ▲—— ———, thymine incorporation by *E. coli* strain 15T$^-$ under identical conditions giving normal growth.

Fig. 4. Growth and thymine incorporation by various *Escherichia coli* strains in liquid minimal medium containing 5 μg. (0·74 μc.) tritiated thymine/ml. Strain 15T$^-$: ○————, growth; △—— ———, thymine incorporation. Strain 15-1B: ●————, growth; ▲—— ———, thymine incorporation. Strain 15T$^-$-3: ◑————, growth; ◭—— ———, thymine incorporation. Strain 15T$^-$-3, but with the thymine added after 2 hr of prototrophic growth (at arrow): ◐————, growth; ◭—— ———, thymine incorporation.

was, nevertheless, extended to include cell homogenates to determine whether enzyme inducibility (reported by Rachmeler, Gerhart & Rosner, 1961, for prototrophic *Escherichia coli*) was a property of thymine-less *E. coli*. Table 2 summarizes the results. Thymidine phosphorylase activities of the mutants fell between the extremes established by wild-type strains. Of special interest is the identical thymidine phosphorylase activity of thymine-grown and thymidine-grown strain 15T$^-$.

The reaction in the reverse direction was weak. Dialysed homogenates from strains 15T$^-$ and 15-1 were compared: 0·5 ml. was mixed to a volume of 1·2 ml. in buffer containing 2·4 μmole (7·4 μc.) tritiated thymine and 1·0 μmole deoxyribose-1-phosphate at 38° and assayed after 1 and 10 hr incubation. Strains 15T$^-$ and 15-1 formed, respectively, 0·050 μmole and 0·040 μmole thymidine/mg. homogenate protein after 1 hr, and 0·090 μmole and 0·092 μmole thymidine/mg. protein after 10 hr.

Nucleoside transdeoxyribosidase and dihydrofolic reductase. The transdeoxyribosidase assays were carried out as described above except that 0·5 μmole deoxyriboside replaced the deoxyribose-1-phosphate. Incubation was for 1 hr and, in some experiments, for 6 hr. No thymidine was formed with deoxyadenosine, although spots on the chromatograms appeared at the adenine position.

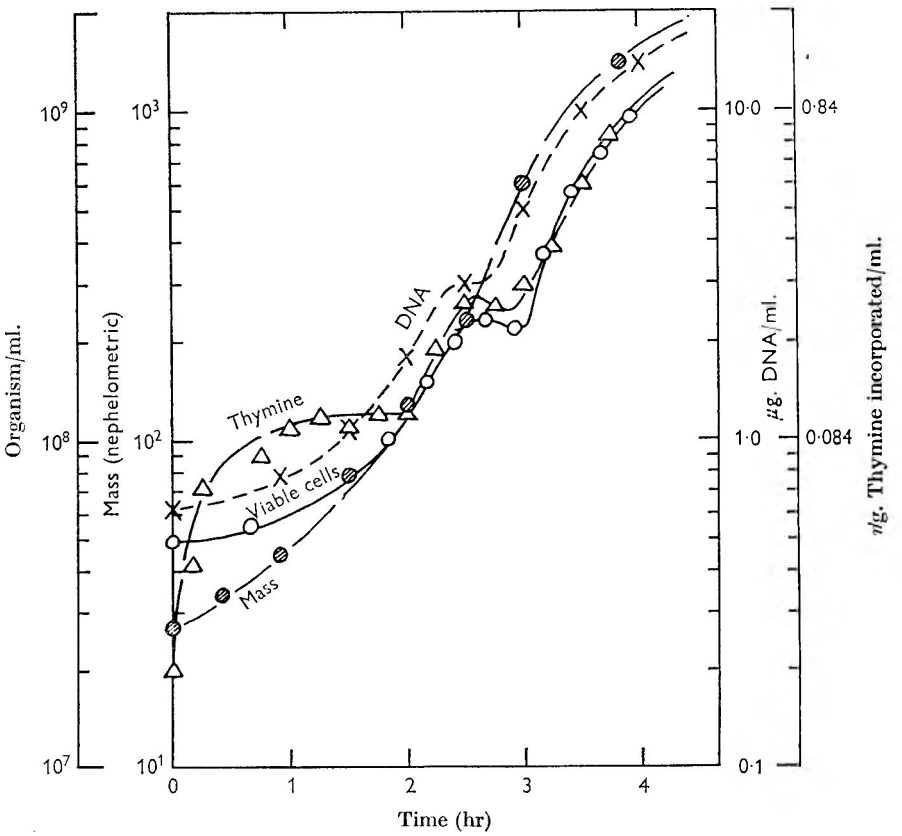

Fig. 5. Response of *Escherichia coli* strain 15-1 in liquid minimal medium containing 30 μg. (4·4 μc.) tritiated thymine/ml.

Apparently the sugar moiety was removed but not transferred to thymine. Moreover, no measurable thymidine was formed with deoxyguanosine or deoxycytidine. The only measurable thymidine formation was with deoxyuridine; strain 15T⁻ formed 0·08 μmole thymidine and strain 15-1 formed 0·10 μmole thymidine/mg. homogenate protein in 1 hr. Thus the difference in thymine incorporation did not reflect a difference in transdeoxyribosidase activity. Further, no correlation between dihydrofolic reductase activity and thymine incorporation was observed (Table 2).

Effect of deoxyribonucleosides and ribonucleosides upon growth. Although deoxyuridine did not replace thymine, it aided in its utilization (Fig. 6). The effect upon diphasic growth was to increase the first growth phase. The effect also occurred on thymidine-grown cultures. Deoxycytidine was the only other deoxyriboside with these properties, but it was less effective (Fig. 6).

Yeast extract (0·2%) in plating agars containing thymine gave low yields whilst those agars containing thymidine yielded numbers in agreement with microscopic counts. Plating agars without yeast extract, for example minimal agar, yielded identical numbers whether thymine or thymidine was used, and the numbers

Table 2. *Enzyme assays on cell homogenates*

Homogenate from strain	Dihydrofolic reductase (mμmole substrate converted/min./mg. protein)	Thymidylate synthetase (mμmole substrate converted/min./mg. protein)	Thymidine phosphorylase (mμmole thymine formed/min./mg. protein)	
			Thymine-grown cells	Thymidine-grown cells
Aerobacter aerogenes NCTC 418				
t	4·6	0·20	—	—
t23	4·7	0·0	—	—
t23A	—	< 0·03	—	—
t23B	4·6	0·0	—	—
t23-1	—	0·23	—	—
t23B-7	—	< 0·03	—	—
Escherichia coli				
15	3·0	0·37	14	120
15-1	3·6	0·0	46	120
15-1B	—	0·0	20	80
15-1-1	—	0·35	—	—
15T⁻	3·1	0·0	65	65
15T⁻-3	—	0·10 and 0·04*	—	—
15T⁻-6	—	0·36	—	—

* Prototrophic growth and growth in 5 μg. thymine/ml., respectively (see text).

agreed with the microscopic count. The inhibitors in yeast extract may be certain ribonucleosides. On minimal agar containing thymine, cytidine and uridine decreased the efficiency of plating of strain t23. The colonies that developed were relatively resistant to inhibition by yeast extract with thymine. (Adenosine and guanosine were less inhibitory than cytidine and uridine.)

Cohen & Barner (1956, 1957) observed that uracil and cytidine inhibited growth of strain 15T⁻. Strong inhibition by uridine and cytidine was unique to the aminopterin-derived mutants; the low thymine requirers (double mutants) derived from them, like strain 15T⁻, were relatively resistant (Table 3). The same response occured, though to a lesser degree, when yeast extract was substituted for uridine or cytidine (Table 3).

Revertants. Heavy seeding of the thymine-less auxotrophs on minimal agar (no thymine) permitted collection of revertants to thymine independence. These were streaked to ensure purity, and stock cultures were prepared on minimal agar. *Aerobacter aerogenes* strain t23 and *Escherichia coli* strain 15-1 gave rise to revertants indistinguishable from the respective wild types, growing normally in minimal medium and incorporating thymine poorly. These revertants are represented in Tables 1 and 2 by strains t23-1 and 15-1-1. The *A. aerogenes* double mutant, strain t23B, gave rise to revertants of two classes. The majority were indistinguishable from a wild type. A few, represented by strain t23B-7, increased in mass at the usual

rate in minimal medium, but increased in number very slowly, the number of viable elongated organisms attaining only 10^8/ml. Growth was normal in 1 μg. thymine/ml. Thymidylate synthetase activity was measurable but very low (Table 2).

When inoculated into minimal medium strain t23A underwent an immediate increase in mass as the cells elongate. This continued for 3–4 hr with no loss in viable count, sometimes with a slight increase, and if the medium contained no added thymine a sharp decrease of viability then took place. But if the medium contained 1 μg./ml. thymine, the culture 'recovered' after a slight loss of viability

Table 3. *Inhibition of growth in thymine by pyrimidine ribonucleosides and by yeast extract*

Strain	Titre on thymine-yeast extract agar/titre on enriched agar	Titre on thymine-cytidine-minimal agar/titre on enriched agar	Titre on thymine-uridine-minimal agar/titre on enriched agar
15T$^-$	1×10^{-2}	3×10^{-1}	5×10^{-1}
15-1	3×10^{-5}	1×10^{-4}	4×10^{-5}
15-1B	1×10^{-4}	4×10^{-1}	2×10^{-1}
15H$^-$T$^-$	2×10^{-3}	1×10^{-1}	1×10^{-2}
CT$^-$	8×10^{-1}	8×10^{-1}	4×10^{-1}
t23	7×10^{-4}	4×10^{-5}	1×10^{-5}
t23B	3×10^{-2}	8×10^{-1}	3×10^{-1}

The composition of enriched agar and minimal agar has been given. Thymine-yeast extract agar is enriched agar in which 25 μg. thymine/ml. has substituted for the thymidine. Cell titres on enriched agar and on thymine-minimal agar were identical and agreed with microscopic count. Cytidine and uridine concentrations were 2000 μg./ml. and 200 μg./ml., respectively. Enriched agar plates were incubated overnight, minimal agar plates 3 days.

and normal growth ensued. In 5 μg. thymine/ml. the 3–4 hr lag did not occur; the culture grew normally from the outset. A very slight thymidylate synthetase activity was detected (Table 2). New single-colony isolations from strains t23A and t23B-7 yielded cultures with identical traits.

Revertants equivalent to strain t23B-7 were not isolated from the *Escherichia coli* double mutant strain 15-1B. All revertants from the latter strain were indistinguishable from the wild type, showing the low rate of thymine incorporation. Strain 15T$^-$ yielded revertants of two classes. The majority, represented by strain 15T$^-$-6, were indistinguishable from the wild type, possessing the same thymidylate synthetase activity (Table 2), manifesting the same low rate of thymine incorporation, and incorporating approximately the same amount of exogenously supplied thymine/cell (Table 1). A few revertants were unusual; they had attributes in common both with the wild type and with the auxotrophic parent.

Figure 7 shows the growth of revertant strain 15T$^-$-3. In dearth of thymine growth was diphasic, whilst in 1 μg. thymine/ml. growth was continuous. The culture was pure: (1) re-isolations yielded cultures with identical characteristics, and (2) the culture described in the figure, after overnight incubation, served as inoculum for a new culture which then underwent identical sequential growth. After several months' storage at 5° this revertant lost its diphasic growth characteristic. Now it grows equally well with or without thymine. When thymine was present, however, it was incorporated at the same rate as by strain 15T$^-$ (Fig. 4). It is as if the normal

control, which mutually excludes efficient thymine incorporation and high thymidylate synthetase activity, were partially lost, allowing the organisms to adjust freely, in the most economical manner, to either auxotrophic or prototrophic growth.

Crawford (1958) described a revertant (15T⁻R) which incorporated about 30 times

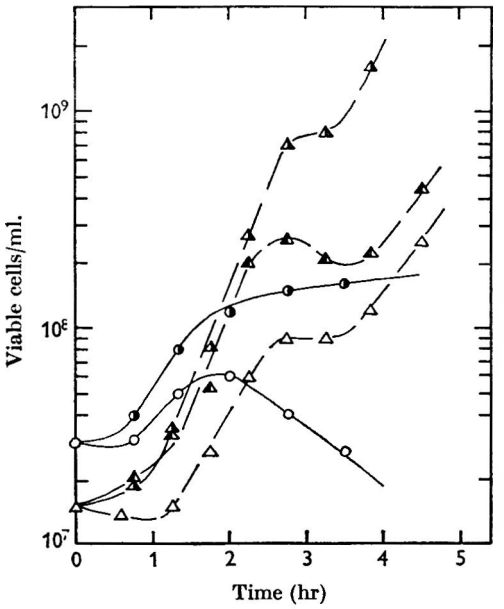

Fig. 6

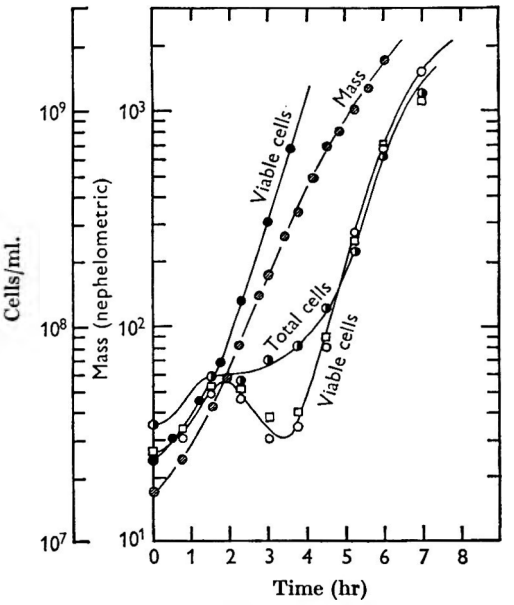

Fig. 7

Fig. 6. Effect of deoxycytidine and deoxyuridine upon growth of thymine-less bacteria in liquid minimal medium. ○————, *Escherichia coli* strain 15-1 in 5 μg. thymine/ml. alone; ◑————, *E. coli* strain 15-1 in 5 μg. thymine/ml. plus 50 μg. deoxyuridine/ml.; △—— ——, *Aerobacter aerogenes* strain t23 in 25 μg. thymine/ml. alone; ▲—— ——, *A. aerogenes* strain t23 in 25 μg. thymine/ml. plus 100 μg. deoxycytidine/ml.; ▲—— ——, *A. aerogenes* strain t23 in 25 μg. thymine/ml. plus 100 μg. deoxyuridine/ml.

Fig. 7. Growth of *Escherichia coli* strain 15T⁻-3. All curves represent assays in liquid minimal medium lacking thymine except curve ●———— where growth was in liquid minimal medium containing 1 μg. thymine/ml. All viable counts were determined with the usual thymidine-yeast extract agar except in curve □———— where minimal agar lacking thymine was employed.

the amount of thymine as did a wild-type strain (strain 15) and about one-fourth the amount as the parent (strain 15T⁻). He did not assay for thymidylate synthetase or describe growth, but it seems likely that his revertant is of the same class as strain 15T⁻-3.

DISCUSSION

Possession of thymidylate synthetase and ability to incorporate exogenous thymine are independent traits, but tend to be mutually exclusive. Bacteria which possess good thymidylate synthetase activity incorporate thymine poorly. Indeed, various devices have been suggested to enhance thymine or thymidine incorporation by wild-type *Escherichia coli* (see, for example, Boyce & Setlow, 1962). Thymine-less

mutants derived by the aminopterin method (for example, strain 15-1) retain the poor thymine incorporation characteristic of the wild type. However, from them, presumably as the result of a second mutation, strains with more efficient thymine incorporation (for example, strain 15-1B) may be selected. The most striking feature of the u.v.-derived 15T⁻, viewed in relation to the wild type and especially in relation to the aminopterin-derived mutants, is not its lack of thymidylate synthetase, but its exceptionally efficient thymine incorporation. Either 15T⁻ is a double mutant also, or it arose as the result of a quite different mutational event. Strain 15T⁻ differs from strain 15-1B and other double mutants: its growth is sustained in lower concentrations of thymine, its thymidine phosphorylase activity is the same whether it is thymine- or thymidine-grown, and it yields a unique class of revertants. Whilst the usual revertants simultaneously regain thymidylate synthetase activity and lose the ability to incorporate thymine efficiently, some from strain 15T⁻ regain thymidylate synthetase but still retain the ability to incorporate thymine.

Poor incorporation of thymine does not seem to be caused by defective permeability because growth is initiated at low thymine concentrations. The following facts are pertinent. Growth is improved by deoxycytidine and deoxyuridine (Fig. 6). Growth is inhibited by cytidine and uridine; strains 15-1 and t23, inefficient incorporators of thymine, yield a thousand times fewer colonies in presence of cytidine or uridine than their respective double mutants and other strains which incorporate thymine efficiently (Table 3). Moreover, high concentration of thymine, in its turn, disturbs growth: not only for strain 15-1 (Fig. 5), but also for strain 15T⁻, though, significantly, to a lesser degree. It is as if thymine metabolism and pyrimidine ribonucleoside metabolism at some point interfere, and much more so in the aminopterin-derived mutants 15-1 and t23. Poor thymine incorporation by 15-1 can be visualized as due to an endogenous inhibitor, the concentration of which can be increased with exogenous cytidine or uridine and decreased or overcome with the respective exogenous deoxyribonucleosides or with thymine at high concentration. The improved thymine incorporation acquired by mutation of 15-1 to 15-1B can be visualized in either of two ways: (1) There are two routes to DNA via thymine, one sensitive and the other relatively resistant to the endogenous inhibitor; the mutation represents a switch from the sensitive to the resistant route. (2) There is one route to DNA; the mutation represents a reduction in the ability to accumulate inhibitor.

Breitman & Bradford (1964) suggested that the more efficient incorporation of thymine by some auxotrophs is due to a greater production of deoxyribonucleosides, allowing deoxyribose transference to thymine 'via transdeoxyribosylation and/or coupled deoxynucleoside phosphorylation mechanisms'.

Although revertant strains t23A and t23B-7 (Table 2) may be considered 'leaky', there is no evidence for leakiness in the aminopterin-derived auxotrophs 15-1 and t23. Thymidylate synthetase was never encountered. Nor was synthesis of DNA in dearth of thymine detected, either by the Dische reaction (Fig. 2), or by the method using D_2O. Furthermore, these bacteria in dearth of thymine (Fig. 2) responded similarly to 15T⁻.

The aminopterin-derived auxotrophs represent a distinctive, stable, and remarkably uniform class of thymine-less bacteria. They emphasize the uniqueness of strain 15T⁻, and also the dual differentiation from the wild type possessed by their double

mutants and by strain 15T⁻. Further, they illustrate well that thymine-less death may occur in the presence of thymine uptake and DNA synthesis. This latter circumstance may be useful to select between two current representations of thymine-less death: (1) unbalanced growth, proposed by Cohen & Barner (1954); (2) mistakes in attempted DNA synthesis as proposed by Maaløe & Hanawalt (1961).

The aminopterin-derived thymine-less bacteria grow very well in thymidylate, a striking difference from their poor growth in thymidine and thymine. The thymidylate is dephosphorylated, probably at the cell surface simultaneously with incorporation of the resulting thymidine. Exogenous thymidine, on the other hand, is rapidly converted to thymine. Apparently the phosphate moiety preserves nucleoside structure. Lichtenstein, Barner & Cohen (1960) proposed separate routes for the utilization of dephosphorylated nucleotide and exogenous nucleoside. The inefficient conversion of thymidine and thymine to DNA by the aminopterin-derived auxotrophs described herein would thus appear to be uniquely associated with the latter route.

The author is indebted to Dr E. Volkin for helpful discussions on pyrimidine metabolism, to Dr W. E. Cohn for advice in paper chromatography, to Dr D. G. Doherty for preparing the tetrahydrofolic acid used in thymidylate synthetase assays, and to Mr L. E. Franklin for skilful assistance early in the work with the many growth studies. The author thanks Professor S. S. Cohen, University of Pennsylvania Medical School, Dr R. B. Setlow, and Dr R. Curtiss for critical readings which led to improvements in the manuscript.

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Deoxyribonucleic Acid Base Compositions among Thermophilic Actinomycetes: the Occurrence of two Strains with Low GC Content

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SUMMARY

Deoxyribonucleic acid (DNA) was extracted from two strains of Thermomonospora, two strains of Thermoactinopolyspora and one thermophilic strain of Streptosporangium. Base compositions were determined from 'melting-temperatures' (Tm) and, in two cases, from buoyant density. DNAs from both Thermoactinopolyspora strains and one Thermomonospora strain were of similar base composition to those previously reported for mesophilic actinomycetes. The other Thermomonospora strain and the Streptosporangium strain, both of which had higher temperature requirements and tolerance for growth, possessed DNA of lower GC content. These findings are discussed in relation to phylogeny, thermophily and the universality of the genetic code.

INTRODUCTION

Increasing importance is currently being given to the base compositions of deoxyribonucleic acids (DNA) as a useful adjunct to taxonomic studies (see Marmur, Falkow & Mandel, 1963, for a recent review). Organisms of the Order Actinomycetales so far studied all possess DNA rich in guanine+cytosine (GC; Table 1). However, these data include no thermophilic actinomycetes. Here we report the base compositions of DNA from five thermophilic actinomycetes; three had % GC values in the range of those given in Table 1, but two possessed very different base ratios.

METHODS

Organisms. The strains of actinomycetes used in this study are listed in Table 2, together with their temperature requirements for growth. Morphological and physiological characterization of strain 5 (*Thermomonospora* sp.) as reported by Craveri & Farina (1963) and Küster & Locci (1963); those of strains 7 (*Thermomonospora* sp.) and 11 (a thermophilic *Streptosporangium* sp.) by Craveri & Manachini (1965); those of strains 6 and 10 (both *Thermoactinopolyspora* sp.) by Craveri & Pagani (1962). The genus *Thermoactinopolyspora* is considered to be closely related to the genus *Thermopolyspora* (Henssen, 1957).

Cultivation of organisms. Cultivation temperatures and media used for each strain are included in Table 2. All strains were grown in two stages on a

Table 1. *DNA base compositions within the genera of the Order Actinomycetales*

Genus	Number of species examined	% GC range	References
Mycobacterium	3	66–72	1, 2
Nocardia	4	68·5–72	1, 3, 4
Streptomyces	17	67–74	1, 3, 4, 5, 6
Micromonospora	1	72	1
Actinoplanes	1	73	4
Streptosporangium	1	68·5	4
Streptoverticillium	1	67	4

1. Belozersky, Shugaeva & Spirin, 1958.
2. Marmur *et al.* 1963.
3. Frontali *et al.* 1965 (buoyant density data; Tm data yielded values up to 78·5 % GC).
4. Jones & Bradley, 1964 (Tm values only given in this paper; % GC obtained by extrapolation in a manner similar to that of Silvestri & Hill (1965), taking *E. coli* = 50 % GC).
5. Marmur & Doty, 1962.
6. Schildkraut *et al.* 1962.

reciprocal shaker. Mycelia were collected by centrifugation after 10–15 hr growth of second stage cultures, thus facilitating subsequent lysis (Frontali, Hill & Silvestri, 1965).

Preparation of DNA specimens and Tm determinations. The procedure of Marmur (1961) was used, lysis of the mycelia being achieved with lysozyme followed by sodium lauryl sulphate. Final isopropanol DNA precipitates were dissolved in a solvent containing 0·01 M-PO_4^{3-} + 0·001 M-EDTA, pH 6·8, specific conductance $1{\cdot}345 \times 10^{-3}$ mho. Melting temperatures (Tm) of DNA preparations were determined as described previously (Frontali *et al.* 1965) and % GC values calculated according to the equation (modified from Marmur & Doty, 1962, for the phosphate solvent used here): Tm = 49·3 + 0·41(GC).

Determination of buoyant density (ρ) values. CsCl buoyant density runs, lasting 20 hr at 42,040 rev./min., were made in a Spinco model E analytical ultracentrifuge.

Table 2. *Organisms, temperature requirements for growth and media used in the present work*

No.*	Name†	Temperature requirements: 37°	Opt. temp. (°)	60°	Cult. temp. (°)	Culture medium‡
5	*Thermomonospora* sp.	Growth	45–50	No growth	47	Ter/1 Suppl.
7	*Thermomonospora* sp.	No growth	55–60	Growth	54	CPL
6	*Thermoactinopolyspora* sp.	Growth	45–50	No growth	47	Ter/1 Suppl.
10	*Thermoactinopolyspora* sp.	Growth	45–50	No growth	47	Ter/1 Suppl.
11	*Streptosporangium* sp.	No growth	50–60	Growth	52	CPL

* Number in the collection held at the Istituto di Microbiologia Agraria e Tecnica.
† According to Craveri & Manachini (1965).
‡ Ter/1 Suppl.: Soya flour, 5 g.; unrefined maltose, 20 g.; yeast extract, 2 g.; peptone, 10 g.; tap water, 1 l.; pH 7·2.
CPL: Meat extract, 5 g.; yeast extract, 1 g.; distilled water, 1 l.; pH 7·2; supplemented with peptone (15 g.) for strain 7 or Tryptone (10 g.) for strain 11.

Bacteriophage LP7 DNA was used as reference (1·740 g./cm.^{3}). % GC was calculated from ρ according to the equation: $\rho = 1{\cdot}660 + 0{\cdot}098(GC)$; (Schildkraut, Marmur & Doty, 1962).

RESULTS

Tm values and calculated % GC values of the DNA preparations are given in Table 3. The Tm values for both *Thermoactinopolyspora* sp. and for one *Thermomonospora* sp. (strain 5) fall within the range of Tm values, in the same solvent, previously reported for mesophilic streptomycetes (79·5–81·5°; Frontali *et al.* 1965). The other *Thermomonospora* sp. (strain 7) and the thermophilic *Streptosporangium* sp., both of which have higher temperature requirements and tolerance for growth than the three previous strains (Table 2), gave much lower Tm values.

Table 3. *Tm, buoyant density and % GC values of the DNA preparations*

No.	Name	Tm (°)	% GC from Tm	ρ g./cm.^{3}	% GC from ρ
5	*Thermomonospora* sp.	79·7[a]	73·9	—	—
		79·5			
7	*Thermomonospora* sp.	67·3	43·8	1·703	44
6	*Thermoactinopolyspora* sp.	81·0	77·4	—	—
10	*Thermoactinopolyspora* sp.	79·7	74·1	—	—
11	*Streptosporangium* sp.	71·1[b]	53·7	1·708	49
		71·5			

Notes. Tm measured in 0·01 M-PO_4^{3-} + 0·001 M-EDTA; *a* two determinations of the same DNA preparation; *b* two DNA preparations. % GC values (col. 4) calculated from Tm = 49·3 + 0·41 (GC) (modified from Marmur & Doty, 1962). ρ values (col. 5) relative to bacteriophage LP 7. DNA = 1·740 g./cm.^{3}; % GC values (col. 6) calculated from $\rho = 1{\cdot}660 + 0{\cdot}098(GC$; Schildkraut *et al.* 1962).

Since this result might have been due to the presence of an unusual base in the DNA, substituting for either adenine, thymine, guanine or cytosine, the two DNA preparations were further examined for their buoyant density in ultracentrifugation. These results are also included in Table 3. The buoyant density result for strain 7 confirms very closely the Tm result, whereas the difference of 4·7 % GC between values calculated from Tm and from buoyant density for strain 11 is comparable with the systematic differences previously observed (Frontali *et al.* 1965). Both Tm and buoyant density values indicate that these two DNA preparations had unusually low GC contents relative to the three thermophilic actinomycetes and other members of Actinomycetales.

DISCUSSION

The present results raise a series of questions about the phylogeny of thermoactinomycetes and the universality of the genetic code. It is generally accepted that bacteria with widely different DNA base compositions are phylogenetically heterogeneous (Marmur *et al.* 1963; Sueoka, 1964: Silvestri & Hill, 1964). According to this view, independent origin from the other actinomycetes would be postulated for the two thermophilic strains found here, from Tm and buoyant density measurements, to possess DNA of unusually low GC content, and they must consequently represent a striking case of morphological evolutionary convergence. We have

recently described (Silvestri & Hill, 1965) another situation of morphological resemblance between bacteria with very different DNA base compositions. Gram-positive catalase-positive cocci had base ratios either in the range 31–36 % GC or in the range 69–75 % GC. However, it was not difficult to imagine that morphological convergence had occurred and that the coccus form, the most simple of morphological forms, had been acquired independently by bacteria of diverse origin. Moreover, taxometric studies had also revealed two distinct groups (Hill, 1959; Hill *et al.* 1965), which corresponded to the base-composition groups. To propose that strains 7 and 11 are of independent origin from the other actinomycetes, and have come to resemble the latter through morphological convergence, meets with greater difficulty in view of the greater morphological differentiation of such organisms. Moreover, Weed (1963), Gause *et al.* (1964) and De Ley (1964) claim to have found induced mutants of other bacteria with DNA base ratios different (in some cases widely different) from their parental strains, thus suggesting the possibility that organisms with different DNA base ratios may have, nonetheless, a common origin.

From the morphological and physiological characters taken into consideration by taxonomists of actinomycetes, the two thermophilic strains of low GC content are similar to corresponding mesophilic strains (Craveri & Manachini, 1965). This similarity, together with considerable differences in DNA base composition, is difficult to understand in terms of the universality of the genetic code, yet may find an explanation in terms of the degeneracy of the code. DNA base compositions of 70–75 % GC on the one hand and 45–50 % GC on the other, suggest that the proteins must also be very different. Thus it is surprising to find identical structures of such relative complexity as the fruiting bodies of actinomycetes. However, since the code is degenerate (Crick, 1963) the hypothesis could be advanced that, in the case of strains 7 and 11, more AT-rich triplets have been selected to code for the same or similar proteins than are coded for by synonymous, but GC-rich, triplets in other actinomycetes. This hypothesis could be tested experimentally by comparing, proteins from both these thermophilic actinomycetes and mesophilic strains.

Another interesting question is whether the low values of % GC found for strains 7 and 11 are connected in some way with their greater thermophily (Table 2), for it is interesting to note that two other thermophilic bacterial species have also been reported to possess DNA base ratios similar to those found here for strains 7 and 11 (*Bacillus stearothermophilus*, 44 % GC, Marmur & Doty, 1962; *Clostridium nigrificans*, two strains, 45 % GC, Saunders, Campbell & Postgate 1964).

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Carotenoids in Mutants of *Verticillium albo-atrum*

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SUMMARY

An orange mutant (M 5) obtained from a wild-type isolate of *Verticillium albo-atrum* by u.v. irradiation was found to contain phytoene, β-carotene, γ-carotene, neo-lycopene A, lycopene, neurosporaxanthin and four unidentified pigments. It is suggested that this simultaneous production of several carotenoid pigments by a hitherto colourless fungus may be regarded as a de-repression leading to the synthesis of a specific precursor in the pathway of carotenoid synthesis. Ultraviolet irradiation of M 5 gave a number of secondary colour mutants that were again analysed: M 5.1, M 5.3 and M 5.4 contained the same pigments as the original M 5 but in widely differing amounts. M 5.2 was colourless like wild type and contained only phytoene. M 5.5 formed a hitherto undetected pigment, torulene, lacked neo-lycopene A and lycopene and produced very little neurosporaxanthin. M 5 and M 5.5 were further investigated over an extended growth period in order to elucidate the sequence of carotenoid formation. The results are discussed in relation to possible hypotheses of carotenoid biosynthesis.

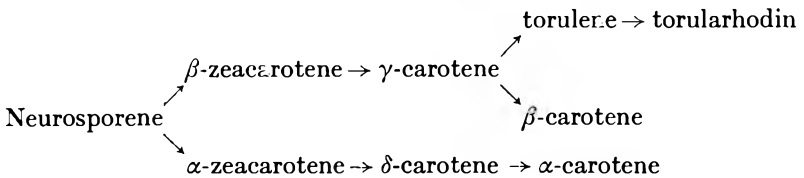
INTRODUCTION

The pathway leading to the synthesis of carotenoids is far from being completely understood. It has been suggested that since two molecules of farnesylpyrophosphate (C_{15}) form squalene (C_{30}), two molecules of geranylgeranylpyrophosphate (C_{20}) could give rise to carotenoids (C_{40}). Similar reactions to those occurring during squalene formation may take place terminating in the formation of lycopersene, its C_{40} homologue. Lycopersene by dehydrogenation could give rise to phytoene and finally carotenoids. In support of this, Grob & Boschetti (1962) identified lycopersene in *Neurospora crassa*, but later Davies, Jones & Goodwin (1963) could not find this compound even though they worked with the same strain. Moreover, most workers have failed to demonstrate the presence of lycopersene in nature, e.g. Mercer, Davies & Goodwin (1963), Beeler, Anderson & Porter (1963), Anderson & Porter (1962), and this has led to an alternative suggestion that the formation of phytoene occurs directly from two molecules of geranylgeranylpyrophosphate. More recently, however, Nusbaum-Cassuto & Villoutreix (1965) have been able to obtain as much as 50 μg. of lycopersene per kg. of fresh white carrots.

As regards the subsequent production of carotenoids from the C_{40} precursor there are two general hypotheses. Porter & Lincoln (1950) have proposed the following pathway of dehydrogenation from phytoene, based on the results of a study on various tomato crosses:

Phytoene → Phytofluene → ζ-carotene → neurosporene → lycopene.

At that time, the exact structures of the intermediates were not known, but more recently results obtained by Anderson, Norgard & Porter (1960) feeding [^{14}C] mevalonate to ripening tomatoes have given support to the above pathway. Further, Beeler & Porter (1962) showed that preparations of tomato plastids could convert phytoene to phytofluene. Earlier Zechmeister & Koe (1954) had reported the isolation of certain cyclic carotenes as a result of the dehydrogenation of phytoene and phytofluene by *N*-bromosuccinimide. There seems to be more support for the formation of cyclic carotenes through neurosporene rather than through lycopene since the intermediates α- and β-zeacarotene have been identified in maize (Petzold, Quackenbush & McQuistan, 1959), in *Rhodotorula glutinis* (Simpson, Nakayama & Chichester, 1964) and the structure of β-zeacarotene determined by Rüegg *et al.* (1961). The proposed scheme is as follows:



In fungi, however, an alternative hypothesis to that of Porter and Lincoln has been proposed, viz. that the C_{40} polyenes could be derived independently from a common precursor. Goodwin (1952), working with *Phycomyces blakesleeanus*, first suggested this when he reported that β-carotene was not formed at the expense of the phytoene series. Later, preliminary kinetic work by Goodwin and co-workers (Davies, Villoutreix, Williams & Goodwin, 1963 suggested that β-zeacarotene, phytofluene, ζ-carotene and neurosporene were formed from phytoene. Results obtained by Zalokar (1954) with *Neurospora crassa* under different conditions of light and oxygen in which ζ-carotene, neurosporene and lycopene all appeared simultaneously support this second hypothesis.

Mutant studies have been used by several workers to examine the biosynthetic pathway of carotenoids in certain fungi. Bonner, Sandoval, Tang & Zechmeister (1946) investigated seven u.v.-induced mutants of the red yeast *Rhodotorula rubra* and their results are consistent with the first (Porter–Lincoln) hypothesis. Villoutreix (1960) worked on u.v. mutants of another red yeast, *R. mucilaginosa*, and concluded that his results could not support the first but the second hypothesis. Haxo (1952), with mutants of the red bread mould *Neurospora crassa*, obtained results that agree equally well with both hypotheses.

In preliminary notes, Valadon & Heale (1964, 1965) reported on an investigation the carotenoids of an orange coloured u.v. mutant (M 5) of *Verticillium albo-atrum*. This was apparently the first record of the simultaneous appearance of several carotenoids in a hitherto colourless fungus. In the present work a number

of new colour mutants have been analysed in order to investigate the biosynthetic pathways of carotenoid production in this fungus.

In the study of coloured mutants of various organisms, changes have been reported in the balance of carotenoid synthesis. Villoutreix (1960) found that some of his mutants of *Rhodotorula mucilaginosa* had the same pigment composition but that the amounts varied. Three mutants of *R. rubra* obtained by Bonner *et al.* (1946) also gave the same picture and they found that these mutants could not be interpreted in any simple way. It has been suggested that the different amounts of carotenoids may reflect alterations in the timing of the various syntheses, and, therefore, two of the mutants in the present work were studied over an extended growth period.

METHODS

Ultraviolet mutants. The wild-type isolate of *Verticillium albo-atrum* Reinke & Berthold used was originally isolated from wilted lucerne in Lincolnshire in 1962. The u.v. irradiation was done with a sterile distilled water suspension of conidia in an open Petri dish at a distance of 20 cm. from a 2537 Å 'Hanovia' bactericidal tube model 11 (with ozone reducing filter). An orange-pigmented colony, designated M 5, developed on plates of complete media spread with conidia irradiated at a dosage allowing for approximately 5% survival. Second-order mutants were produced in a similar manner using M 5 as the parent source of conidia and they were selected by visual inspection of complete medium plates.

Extraction and identification of carotenoids. Cultures were grown on Wickerham's (1951) MYGP liquid medium on a reciprocating shaker at 20° in the light. The pigments were extracted from 10-day-old cultures with methanol and transferred into diethylether by addition of water. The ethereal layer was evaporated under reduced pressure at 30° and then taken up in methanol. This was saponified with 60% aqueous KOH overnight (Goodwin, 1955) and extracted with ethyl ether. The ethereal layer was washed with tepid water, freeze dried and the pigments taken up in about 2 ml. *n*-hexane. This was then chromatographed on a magnesium oxide + 'Celite' column (1 + 1, v/v) and developed by washing with increasing concentrations of ether in *n*-hexane. In some of the cultures, the top brown orange band was strongly adsorbed to the powder and could be eluted by 0·5% glacial acetic acid in methanol.

The first colourless fraction which was eluted with purified *n*-hexane contained sterols, and these were removed according to the method of Mercer *et al.* (1963). Phytoene was identified in this fraction by means of its spectrum in *n*-hexane ($\sim$ 272, 284, $\sim$294 mμ). The coloured bands eluted with ether in *n*-hexane were evaporated to dryness, taken up in *n*-hexane and the spectra estimated using a Unicam SP 500 spectrophotometer. The absorption spectra of the carotenoids were also measured in carbon disulphide and in benzene, while those of the fraction identified as neurosporaxanthin were also measured in acid methanol (1% acetic acid) and in alkaline methanol (0·5% KOH). These were then compared with spectra of known carotenoids and their probable natures determined. To identify β-carotene, γ-carotene, neo-lycopene A and lycopene, respectively, a pure pigment was mixed with the unknown, co-chromatographed on activated alumina (Goodwin, 1954) eluted with ether in *n*-hexane and shown to be one and the same pigment when

they ran as one band. In the case of torulene co-chromatography was carried out on a calcium hydroxide column using benzene as developer and for neurosporaxanthin on a sucrose column using 10 % methanolinhexane as developer. Pure β-carotene, γ-carotene, neo-lycopene A and lycopene were obtained from *Calendula officinalis* (Goodwin, 1954); torulene from *Rhodotorula rubra* (Le Rosen & Zechmeister, 1943) and neurosporaxanthin from *Neurospora crassa* (Zalokar, 1957).

RESULTS

M 5 was found to have phytoene, β-, γ-carotene, neo-lycopene A, lycopene, neurosporaxanthin and four unidentified bands, two of which were in too small a quantity to be identified further, and two other red and brown bands.

From the further irradiation of M 5 a number of secondary mutants were obtained and five of these (M 5.1 to M 5.5) were differently coloured and were analysed with respect to their carotenoid pigments (Table 1).

Table 1. *Polyenes (μg./g. dry weight) of wild type and mutants of Verticillium albo-atrum after 10 days in culture*

	Appearance						
Pigments	Wild type, colourless	M 5, orange	M 5.1, dark-orange	M 5.2, colourless	M 5.3, red-orange	M 5.4, brown-orange	M 5.5, pink-red
Phytoene	28·4	20·1	12·3	21·0	18·9	16·7	26·5
β-Carotene	—	23·4	28·6	—	21·2	6·8	24·2
γ-Carotene	—	66·4	54·7	—	180·5	66·9	43·3
Neo-lycopene A	—	11·09	Trace	—	Trace	24·1	—
Lycopene	—	121·9	257·4	—	128·1	13·5	—
Torulene	—	—	—	—	—	—	195·0
Brown band	—	11·3	15·6	—	24·2	26·3	Trace
Red band	—	22·4	18·7	—	22·3	18·8	Trace
Neurosporaxanthin	—	178·5	255·5	—	261·4	193·0	5·0

Three coloured secondary mutants (M 5.1, M 5.3, M 5.4) contained the same carotenoids as the original mutant M 5 but in widely differing amounts. One mutant, M 5.5, formed a hitherto undetected pigment, torulene, lacked neo-lycopene A and lycopene and produced very little neurosporaxanthin. M 5.2 was very much like the wild type in its polyene content; it was colourless, containing only phytoene at similar amounts to that found in all the mutants and in the wild type.

The pigments of M 5 were analysed over a period of 21 days' growth (Fig. 1). Phytoene was estimated at 21·7 μg./g. dry weight at 6 days, dropped to a minimum of 10·04 μg. after 18 days and rose to 17·85 μg. at 21 days. β-Carotene, neo-lycopene A and the two unidentified bands were all present at a concentration of approximately 20 μg./g. and did not vary very much over the period studied. γ-Carotene, lycopene and neurosporaxanthin were at a minimum after 6 days, rose to a peak at the 15th day and fell slightly after 21 days. The maximum for neurosporaxanthin was 245·11 μg./g. after 18 days.

The pigments of M 5.5 were analysed over 18 days' growth (Fig. 2). The phytoene and β-carotene amounts were very much like those observed in M 5. The two un-

identified bands were present as traces up to the twelfth day and thereafter increased slightly to approximately 4 μg./g. Neurosporaxanthin, however, was not found until the tenth day, and then only in traces, but later this reached a maximum of 25·24 μg. by the sixteenth day. γ-Carotene was fairly high, 53·70 μg. on the fourteenth day, but never as high as in M 5 (150 μg. maximum). Torulene was the one pigment found in large amounts and it showed a similar curve to that of neurosporaxanthin for M 5, the peak in this case being 221·26 μg. on the fourteenth day.

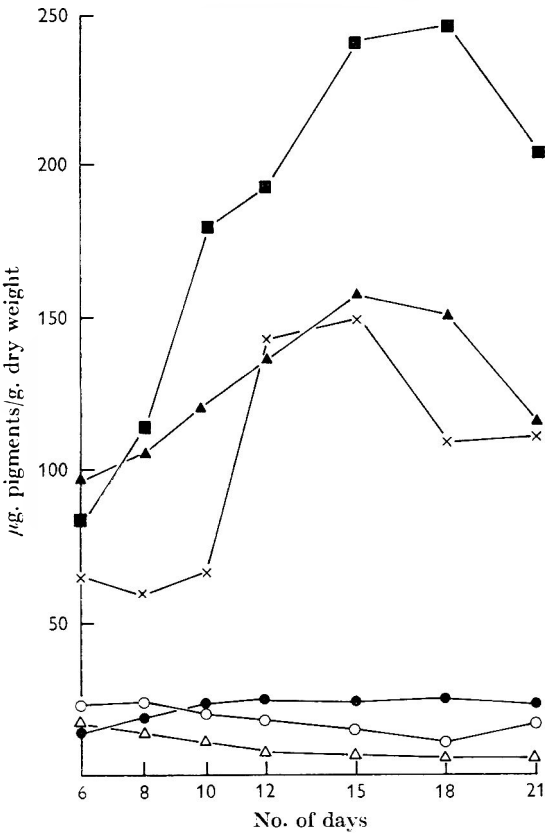


Fig. 1

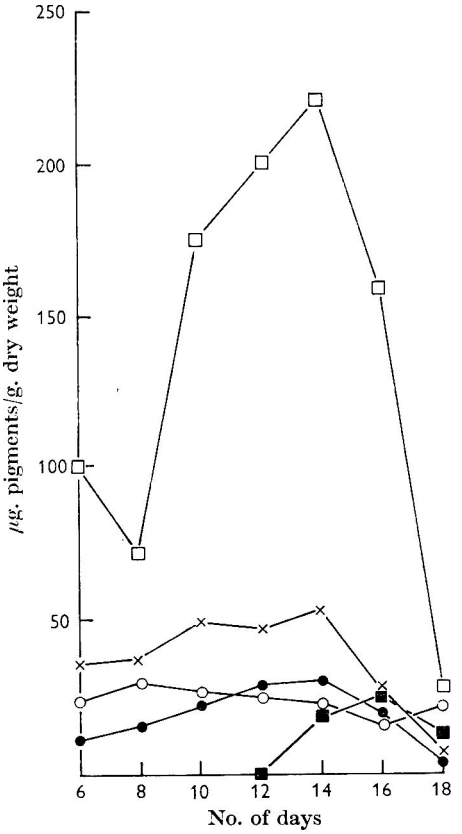


Fig. 2

Fig. 1. Changes in amounts of the different polyene pigments with age of the mutant M 5 of *Verticillium albo-atrum*. Phytoene, ○—○; β-carotene, ●—●; γ-carotene, × — × ; neo-lycopene A, △—△; lycopene, ▲—▲; neurosporaxanthin, ■—■.

Fig. 2. Changes in amounts of the different polyene pigments with age of the mutant M 5.5 of *Verticillium albo-atrum*. Phytoene, ○—○; β-carotene, ●—●; γ-carotene, × — × ; torulene, □—□; neurosporaxanthin, ■—■.

DISCUSSION

The simultaneous production of several carotenoid pigments by mutant M 5, obtained in one step from a colourless wild-type *Verticillium albo-atrum*, is interesting and its interpretation may have some significance in our understanding of the genic regulation of carotenoid synthesis. It becomes necessary to explain the complete absence of carotenoids in the wild type, and also, since we assume that the mutation was operative at a single site, to explain how it could result in the formation of

numerous pigments in M5. We suggest the simplest explanation is that the mutation occurred at a repressor site, which functions normally in the wild type to repress synthesis of a carotenoid pre-cursor 'P'. The resulting loss of activity of the repressor in M 5 thus led to the production of 'P' (de-repression) which presumably then induced formation of the enzymes necessary for carotenoid synthesis. This de-repression is now known to be readily reversible, since subsequent work has shown that colourless mutants, indistinguishable from wild type and similar to M 5.2, are frequently obtained by u.v. irradiation or nitrous acid treatment of M 5 conidia (Heale, unpublished observation).

The study of M 5 over the growth period was made to establish whether its sequence of carotenoid synthesis reflected the variations of pigment composition shown by those mutants with a similar pattern of carotenoids, i.e. M 5.1, 5.3 and 5.4. If M 5.1 is compared with M 5 over 17 days when the amounts of phytoene and β-carotene are almost the same, one finds that γ-carotene is less abundant in M 5.1; neo-lycopene A and lycopene are more abundant, while neurosporaxanthin is more or less the same in both. The lycopene level of M 5 over 21 days is never as high as that of M 5.1. Similarly, M 5.3 and 5.4 can be compared with M 5 and it can be seen that the differing amounts of carotenoids in these secondary mutants cannot be simply explained as being due to alterations in the sequence of their formation as observed in M 5.

B. H. Davies has shown that lycopene is found in young cultures of *Rhizophlyctis rosea*, while both lycopene and γ-carotene are found in older cultures (quoted by Goodwin, 1965). In M 5.5 (Fig. 2) torulene appears to replace lycopene found in M 5, suggesting that these two carotenoids may be linked in a synthetic pathway; there is evidence that γ-carotene is the precursor of torulene (Kayser & Villoutreix, 1961; Simpson *et al.* 1964) and both lycopene and γ-carotene have neurosporene as their precursors. The presence of neurosporaxanthin in this mutant M 5.5 was interesting in that its synthesis was markedly delayed, only appearing after 14 days. Haxo (1952) obtained a somewhat similar result with mutants of *Neurospora crassa* which showed altered polyene composition, but in one of these there was a complete pigment block leading to elimination of neurosporaxanthin.

If one accepts the Porter–Lincoln hypothesis, one might expect that as phytoene is the precursor, it would be found in larger amounts when other carotenoids are not formed, for example, in the wild-type and in M 5.2. It seems, therefore, that the similar amounts of phytoene in the wild-type and mutants do not lend support to this hypothesis. However, precursor-product relationships are not always reflected in their quantities, or even in the detectability of the compounds involved (D. G. Anderson, personal communication) and so conclusions based on this type of evidence must be tentative only. On the other hand, the expected effect was shown to a certain extent by the two mutants M 5 and M 5.5 (when they were analysed over an extended period of growth) in that a slight fall occurred in the phytoene concentration during the synthesis of neurosporaxanthin. Zalokar (1954), working on the biosynthesis of carotenoids in *Neurospora crassa*, obtained a similar result and suggested that phytoene might be the precursor of neurosporaxanthin.

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Temperate Phages Influencing Lipase Production by *Staphylococcus aureus*

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SUMMARY

Phages capable of changing the production of the 'Tween'-splitting enzyme by lysogenic conversion of *Staphylococcus aureus* strains were isolated from lysogenic strains within the phage type 52/52A/80/81 complex.

Phages isolated from TW$^-$ strains were able to block the production of the 'Tween'-splitting enzyme when they lysogenized TW$^+$ strains. When the TW$^+$ receptor strains were originally lysogenic, double lysogenization occurred. This change in the 'Tween' reaction was shown to be an example of lysogenic conversion. Phages isolated from TW$^+$ strains were found able, in some instances, to change TW$^-$ strains to TW$^+$ strains on lysogenization. This change in reaction was found to be due to loss of the converting phage carried by the recipient strains by prophage substitution. Where prophage substitution did not occur and the strains became doubly lysogenic, the strains remained TW$^-$.

In these experiments a change of the typing pattern was found after lysogenization. The phage type of the lysogenized strain became similar to that of the donor strain. Non-typable strains were found, doubly lysogenic for phages which between them blocked all the reactions to the typing phages employed. The significance of these findings is discussed, especially the use of lysogenized strains in experiments designed to investigate the role of 'Tween' negavity as a virulence factor.

INTRODUCTION

Previous investigations of staphylococcal bacteraemia cases (Jessen *et al.* 1963) indicated that *Staphylococcus aureus* strains which did not produce a 'Tween'-splitting enzyme (TW$^-$) might be more virulent than those which did produce the enzyme (TW$^+$). Further investigations into factors influencing this enzyme production by *Staphylococcus aureus* strains showed that it was possible by lysogenization to make TW$^+$ strains TW$^-$ (Rosendal, Bülow & Jessen, 1964).

It was concluded, from these experiments that a lysogenic conversion took place, since all colonies made TW$^-$ were lysogenic, and loss of the prophage caused a reversion to 'Tween' positivity. Furthermore, the converting property of the phages was not influenced by the 'Tween'-splitting property of the last strain on which the phages were propagated.

The phages with converting properties were all isolated from TW$^-$ strains lysed only by typing phage 80, whereas the sensitivity to phage 81 was blocked. On lysogenization the TW$^+$ receptor strains, which all belonged to phage type 80/81, became TW$^-$ and resistant to phage 81.

The present survey includes further investigations into temperate phages isolated from both TW$^-$ and TW$^+$ strains belonging to the 52/52A/80/81 phage type complex (types 80, 81, 80/81, 52/52A/80 and 52/52A/80/81) and all capable of changing the 'Tween'-splitting properties of sensitive strains by lysogenic conversion or by prophage substitution of converting phages present in the TW$^-$ strains.

METHODS

'Tween'-splitting enzyme was shown on 'Tween' 80/calcium agar plates (Sierra, 1957).

Staphylococcal strains. A list of the lysogenic strains from which the phages were isolated is given in Table 1.

Table 1. *Phage types of the donor strains and their non-lysogenic mutants*

Strain	TW	Phage types		Phage isolated	Serol. group of phage
		RTD	1000 × RTD		
24724	−	80	52/52A/80/47C/52B	+	A
24724	+	80/81/42B/47C	52/52A/80/81/42B/47C/52B	−	
311	−	80	52/80/47C	+	F
311	+	80/81	52/52A/80/81/42B/47C	−	
1543	−	52/52A/80/81/42B/47C	52/52A/80/81/42B/47C/52B	+	A
1543	+	80/81/42B/47C	52/52A/80/81/42B/47C/52B	−	
11352	−	81/47C	81/42B/47C	+	B
11352	+	52/52A/80/81/47C/52B	52/52A/80/81/54/42B/47C/52B	−	
1400	−	81/42B	81/42B	+	B
1400	+	52/52A/80/81/42B/52B	29/52/52A/80/81/42B/52B	−	
327	+	52/52A/80/52B	52/52A/80/52B	+	A
7578	+	52/52A/80/81/42B/47C/52B	52/52A/80/81/42B/47C/52B	+	A
1433	+	81 (42B/47C vw*)	81/42B/47C/52B	+	B

* vw = very weak reaction.

From all the TW$^-$ strains TW$^+$ variants were isolated with a frequency of about 10^{-4}. (Four-hr cultures grown in trypsin broth with 2×10^{-2} M-sodium citrate added were spread on 10 'Tween'/calcium agar plates to give about 5×10^3 colony-forming units per plate. TW$^+$ colonies were easily seen among the great majority of TW$^-$ ones).

Several other TW$^-$ and TW$^+$ strains belonging to phage types 80, 81, 80/81, 52/52A/80 and 52/52A/80/81 were used to investigate the lysogenic effect of the various phages. Some of them were selected as indicator strains for the phages.

Phages were isolated from the strains listed in Table 1, using the chloroform method of Kjems (1955). They were propagated to a titre of about 10^9 ml. as suggested by Blair & Williams (1961). Their antigenic characters were determined by Rountree's method (1949). The phages are designated by the number of their staphylococcal strain of origin followed by a prime sign. Several other phages with lysogenic properties identical to those referred to have been investigated but details are given only of one representative of each group.

Lysogenization was carried out as described by Rountree (1959). The lysogenized cultures were tested for resistance to the lysogenizing phage and for phage produc-

tion by the chloroform method (Kjems, 1955). Phage production was confirmed by applying the replica test (Lederberg & Lederberg, 1952) to living cultures passaged three times through medium containing anti-phage serum. In investigating double lysogeny two different indicator strains, each lysed by only one of the phages in question, were used.

The lysogenized cultures are described in the usual way, e.g. 24724 (311′) is strain 24724 lysogenized with phage 311′ isolated from strain 311.

Phage-typing was done by the method of Blair & Williams (1961). The phages used were those described by Rosendal, Stenderup, Helms & Eriksen (1963).

RESULTS

Relation between lysogeny and the 'Tween' reaction. From Table 1 it is seen that none of the TW$^+$ variants isolated from the TW$^-$ cultures was shown to produce phage. They were easily lysogenized by phages from the parent culture which made them TW$^-$ and blocked all the reactions to the typing phages by which they differed from the corresponding TW$^-$ strain, their phage pattern in most cases being broader.

None of the strains lysed by typing phage 80 was completely resistant to the phages 52 and 52A which were able to lyse them at 1000 × RTD, whereas in the strains not lysed by phage 80, but by phage 81, the block to phages 52 and 52A was complete.

However, some TW$^+$ strains were found to be phage producing (327, 1433 and 7578), and their phages were also investigated.

All the phages referred to in Table 1 must be considered temperate, since they were able to lysogenize.

Effect of lysogenization on phage-typing pattern. Tables 2 and 3 give information about the changes in sensitivity to typing phages which took place after lysogenization with the various phages. The changes caused by phages isolated from TW$^-$ strains are recorded in Table 2, those caused by phages from TW$^+$ strains in Table 3. The results were obtained by lysogenizing a variety of strains within the type 52/52A/80/81 complex. It will be seen that some phages were able to block certain reactions, whereas others remain unchanged, depending upon the phage used. In some cases a gain in sensitivity was achieved. When, for instance, a type 81 was lysogenized by a phage from type 80, the lysogenized culture could be typed as type 80. Phage 1543′ did not block any of the reactions examined.

When TW$^-$ cultures were lysogenized with the phages 327′, 1433′ and 7578′, both TW$^-$ and TW$^+$ representatives, with phage pattern indicating lysogenization, were isolated.

Prophage substitution and double lysogenization. In previous experiments (Rosendal *et al.* 1964) the converting property of the phages was shown by using receptor strains not known to be lysogenic, therefore it could not be shown whether a prophage substitution was responsible for the change of the 'Tween'-splitting property or not. So converting experiments were performed using only strains lysogenized with a demonstrable prophage and phages which were able to lyse or lysogenize these strains (Table 4).

From the results it is evident that a prophage substitution took place only when TW$^-$ strains became TW$^+$ upon lysogenization. When these cultures still retained their

Table 2. *Effect of lysogenization with phages blocking the production of the 'Tween'-splitting enzyme on reaction to typing phages*

Phage	Phage type of donor strain (TW⁻)	Typing phages 52	52A	80	81	42B	47C	52B
24724′	80	×	×	*o*	×	×	×	×
311′	80	×	×	–	×	×	*o*	×
1543′	52/52A/80/81/42B/47C	*o*	*o*	*o*	*o*	*o*	*o*	*o*
11352′	81/47C	×	×	×	*o*	–	–	×
1400′	81/42B	×	×	×	*o*	*o*	×	×

× indicates blocking of sensitivity.
– indicates no blocking of sensitivity.
o indicates gain in sensitivity.

Table 3. *Effect of lysogenization with phages capable of making TW⁻ cultures enzyme producing. Alteration of reaction to typing phages*

Phage	Phage type of donor strain (TW⁺)	Typing phages 52	52A	80	81	42B	47C	52B
327′	52/52A/80/52B	*o*	*o*	*o*	×	×	×	*o*
7578′	52/52A/80/81/42B/47C/52B	*o*	*o*	*o*	*o*	–	–	*o*
1433′	81 (42B/47C vw*)	×	×	×	*o*	*o*	*o*	×

* vw = very weak reaction.
× indicates blocking of sensitivity.
– indicates no blocking of sensitivity.
o indicates gain in sensitivity.

'Tween' negativity even though lysogenized with the phages 327′ or 1433′, they had become double lysogenic and were still carrying their original prophage, as evidenced by resistance to and production of both phages in question and change of phage type.

Both the TW⁺ lysogenic cultures were still lysogenic for the original prophage when they were lysogenized with a phage which made them TW⁻.

An experiment was done to find out whether these phages, derived from TW⁻ cultures, in fact substituted for the converting prophage or whether the TW⁻ lysogenized individuals originated from lysogenization of TW⁺ mutants present in the original culture. In this experiment phage 327′ was used as a representative of the phages which can convert TW⁻ strains. A culture of 311 (TW⁻) in a suitable concentration to give single colonies was grown on 'Tween' agar for 2 hr, so that less than 100 cocci per colony-forming unit might be expected. This plate was used as a master plate for transfer by replica technique to an extract agar plate flooded with 327′ (10^9 phage particles/ml.). After overnight incubation at 30° this plate in turn was transferred to a second 'Tween' agar plate. It was found that 48 out of 66 originally TW⁻ colonies had been converted to TW⁺. One of the converted colonies was cut out of the master plate and transferred to broth with 2×10^{-2} M-sodium citrate added. After growth at 37° for 2½ hr one ml of a 10^{-3} dilution was spread onto 10 'Tween' agar plates, giving a growth of about 5000 colonies per plate.

Table 4. *Presence of prophage(s) in various cultures before and after lysogenization with phages capable of changing the Tween splitting property of the culture*

Strain	Phage type	TW	Lysed by phages					Phage(s) produced	Pro-phage substitu-tion	Double lyso-geniza-tion
			311	1543	11352	327	1433			
311	80	−	−	.	.	+	+	311	.	.
311 (327′)	52/52A/80/52B	+	+	.	.	−	.	327	+	−
311 (327′)	52/52A/80/52B	−	−	.	.	−	.	311, 327	−	+
311 (1433′)	81/47C	+	+	.	.	.	−	1433	+	−
311 (1433′)	47C	−	−	.	.	.	−	311, 1433	−	+
1543	52/52A/80/81/42B/47C	−	−	−	.	+	+	1543	.	.
1543 (327′)	52/52A/80/52B	+	.	+	.	−	.	327	+	−
1543 (327′)	52/52A/80/52B	−	.	−	.	−	.	1543, 327	−	+
1543 (1433′)	81/42B/47C	+	.	+	.	.	−	1433	+	−
1543 (1433′)	81/42B/47C	−	.	−	.	.	−	1543, 1433	−	+
11352	81/47C	−	.	.	−	+	−	11352	.	.
11352 (327′)	52/52A/80/52B	+	.	.	+	−	.	327	+	−
11352 (327′)	NT†	−	.	.	−	−	.	11352, 327	−	+
327	52/52A/80/52B	+	+	.	+	−	.	327	.	.
327 (311′)	80	−	−	.	.	−	.	327, 311	−	+
327 (11352′)	NT	−	.	.	−	−	.	327, 11352	−	+
1433	81 (42B/47C vw*)	+	+	.	−	+	−	1433	.	.
1433 (311′)	47C	−	−	.	.	.	−	1433, 311	−	+

* vw = very weak reaction † NT = non-typable

Only 14 TW+ colonies were found amongst the 50,000 colonies, indicating that the TW+ mutants in the original culture could not account for all the conversions found.

Table 5. *Phage types after lysogenization*

Strain	Before lysogenization	TW	Prophage substitution	Double lysogenization
311 (1543′)	80	–	52/52A/80/81/42B/47C	80/47C
1543 (311′)	52/52A/80/81/42B/47C	–	80/47C	.
1543 (24724′)	52/52A/80/81/42B/47C	–	80	52/52A/80
24724 (311′)	80	–	80/47C	80
24724 (1543′)	80	–	52/52A/80/81	.
1433 (327′)	81 (42B/47C vw*)	+	.	NT†

No change of the 'Tween' reaction. Donor strain and receptor strain have the same 'Tween'-splitting capacity.

* vw = very weak reaction. † NT = non-typable.

TW– strains carrying converting phages could themselves be lysogenized with converting phages from other TW– lysogenic strains (Table 5). Both prophage substitution and double lysogenization occurred. No effect was produced on the 'Tween'-splitting reaction as might be expected since both donor and recipient strains were TW–, but there was an effect on the phage-typing pattern which differed according to the lysogenization, depending on whether prophage substitution or double lysogenization took place. The changes in typing pattern corresponded to the changes expected on the basis of the results presented in Tables 2 and 3. No prophage substitution was observed in strain 1433 (327′), which agreed with the previous finding that prophage substitution did not occur in lysogenic TW+ strains and suggests that the phages carried by TW+ strains were more stably integrated than was the case with the TW– converting phages.

DISCUSSION

The assumption that there might exist a correlation between 'Tween' negativity and virulence originated from investigations of survival or death of about 500 patients with staphylococcal bacteraemia (Jessen *et al.* 1963). So far, however, it has not seemed possible to obtain experimental proof of this hypothesis as the selection of strains would have been too difficult. There would always have been some doubt whether the strains chosen were in fact comparable, they might differ in some unknown properties. But pairs of TW+ staphylococcal strains before and after conversion to 'Tween' negativity do seem to be comparable and may offer information about differences in virulence and in biochemical properties. Now, a further question arises, is the lack of diffusible 'Tween'-splitting enzyme itself a virulence factor, or do some accompanying properties account for the enhancement of virulence?

If experiments showed that conversion to 'Tween' negativity was correlated with an increase of virulence, it would be another example of virulence in bacteria depending upon lysogenic conversion, the first and classical example of which is

Corynebacterium diphtheriae (Freeman, 1951). In the present experiments it did seem that Tween negativity depended on the presence of a prophage, whereas Tween positivity did not. The TW$^-$ strains so far investigated have always been lysogenic, but only some TW$^+$ strains, and lysogeny has not been shown in the TW$^+$ variants of the TW$^-$ strains, although a large number of indicator strains were used. Furthermore, the phages isolated from TW$^+$ strains were only capable of 'converting' by virtue of prophage substitution and cannot be regarded as true converting phages like those carried by TW$^-$ strains.

In some instances the conversion of staphylococci provides a handy tool for isolating variants within a bacterial population. In TW$^-$ cultures the mutants having lost the prophage and therefore having become TW$^+$ were easily detected on the Tween medium, and in lysogenization experiments the lysogenized individuals were easily scored on Tween agar when a change of enzyme production had taken place. However, it must be born in mind that in TW$^-$ cultures lysogenized by phages 327′ and 1433′ the lysogenized individuals did not differ from the non-lysogenized ones unless a prophage substitution had occurred.

The similarity of the phage types of these TW$^+$ variants (Table 1) supports the theory of Rountree (1959) that the parent phage type of the 52/52A/80/81 complex may be type 52/52A/80/81/42B/47C/52B, and that all other phage types within the complex may originate from it by lysogenization with various phages.

The changes of phage type upon lysogenization agreed with those found by other authors (Rountree, 1959; Asheshov & Rippon, 1959; Rountree & Asheshov, 1961) and was understandable in view of what was shown about the outcome of lysogenization (prophage substitution or double lysogeny). Non-typable subcultures were also found in cultures which were doubly lysogenic for phages which between them blocked the reactions to all the typing phages in question. Comtois (1960) also found non-typable subcultures upon lysogenization, and supposed that the immunity to typing phages in these instances 'occurred in blocks'.

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The Taxonomy of certain Thiobacilli

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SUMMARY

Numerical analysis has been applied to the taxonomy of certain Thiobacilli; 54 strains were examined, including both authentic cultures and new isolates, using 48 tests. The groups found corresponded to *Thiobacillus trautweinii*, *T. novellus*, *T. thioparus* and *T. neapolitanus*, and these were very well differentiated with no intermediate organisms. A check of the numerical method indicated that the results were independent of the tests used. A comparison of authentic strains from different laboratories showed their characteristics to be very stable, and any changes were within the error of the test method.

INTRODUCTION

During a study of the autotrophic bacteria responsible for the oxidation of reduced sulphur compounds, difficulty was experienced in identifying the various Thiobacillus species, since many of the criteria given by Parker & Temple (1957) appeared to be unsatisfactory. The most serious problem was that no strain could be found corresponding to *Thiobacillus thioparus* as described by these workers, since all the cultures examined produced polythionates. Similarly, the pH values given were of little use since no differentiation is made between the limits for the initiation of growth and the final pH value attained by the culture.

An examination of the literature of this field indicated that opinions differed on the taxonomy of this group. Some authors considered the genus to be a spectrum of types (Baalsrud, 1954), whilst others recognized distinct species, but these varied according to the author (Vishniac & Santer, 1957; Parker & Prisk, 1953). The latest comprehensive study of this group was made in 1953, and in this instance only the utilization of sulphur compounds was investigated and not all the species were examined. These facts led to the conclusion that a new taxonomic survey of the thiobacilli was required and that as many strains as possible should be investigated using a large number of differential tests.

This would involve the collection of a large amount of data and the programme was planned so that this could be subjected to a numerical analysis of the type described by Sneath (1957). At this point, however, no decision was made as to the method to be used in the final interpretation of the results, as no critical investigation of the numerical methods had been carried out.

A secondary aspect of this programme was the further investigation of the interspecies change which had been reported previously in this genus (Johnstone, Townshend & White, 1961). With a large number of strains under continuous observation, any change would be readily noted, the variants could be tested, and

the magnitude of the change determined. An additional assessment of the stability of these bacterial strains was made by the inclusion in the study of cultures of the same authentic strain from several sources. Since these cultures would have been maintained under different conditions in the various laboratories, any changes should have been obvious when they were examined by the same test scheme.

METHODS

Organisms. All the authentic cultures which could be obtained are given in Table 1 together with the worker or organization who supplied them. The attempt to collect cultures of the same authentic strain from several sources was most successful with *Thiobacillus thioparus*; cultures were obtained from three laboratories.

Table 1. *Authentic strains*

Collection or source	Name	Code number
ATCC 8093	*T. novellus**	a1
ATCC 8158	*T. thioparus**	a7
NCIB 8539	*T. neapolitanus*	b1
NCIB 9113	*T. novellus**	b2
NCIB 8370	*T. thioparus**	b5
P. A. Trudinger	Thiobacillus X (a)	c1
P. A. Trudinger	Thiobacillus X (b)	c2
T. Wiken	*T. trautweinii* (str. 1)	d1
T. Wiken	*T. trautweinii* (str. 2)	d2
F. C. Happold	*T. thioparus**	11
F. C. Happold	*T. thiocyanoxidans* (1)	12
F. C. Happold	*T. thiocyanoxidans* (2)	13
F. C. Happold	*T. denitrificans*†	14
Mrs M. Townshend	—	m1
Miss E. S. Pankhurst	T3‡	p1
Miss E. S. Pankhurst	T4‡	p2
Miss E. S. Pankhurst	T2‡	p3
Miss E. S. Pankhurst	C‡	p4
Miss E. S. Pankhurst	T5‡	p5
T. G. Tomlinson	*T. thiocyanoxidans*	t1
T. G. Tomlinson	*T. thioparus**†	t3

* These strains originated from R. L. Starkey.
† An autotroph could not be isolated from these cultures.
‡ Pankhurst (1934).

Since the number of authentic cultures was small, new strains were isolated from the sources given in Table 2. The variety of sources examined, together with the method of isolation used, also ensured that the strains examined were representative of the group as a whole. After suitable dilution, material from the source was inoculated into liquid and on to solid thiosulphate media of pH values from 5·0 to 7·8. At intervals the liquid enrichment cultures were plated out, and representative colonies selected from the initial and subsequent plates. The strains were purified by at least three single colony isolations.

Although many of the thiobacilli can be readily recognized by the inter-colonial deposition of sulphur, the only criterion used in the selection of isolates was the ability to grow on a thiosulphate plate. This ensured that the extent of the group investigated was not limited by the preconceived principle that all thiobacilli deposit sulphur.

Maintenance of organisms. The strains were maintained on plates of the S6 medium (see below) and subcultured at 21-day intervals. The plates were incubated at 28° until growth had occurred and then stored at 5° for the remaining time interval. Before subculturing, each plate was checked under the microscope for purity and before testing, each culture was plated out on both S6 and nutrient media. The latter plates were examined for atypical growth and any autotrophic strains which grew on the nutrient media were discarded.

Table 2. *New isolates*

Source	Code number of isolates
Barren soil	1A
Marine mud	1B, 2B, 3B, 4B, 6B, 7B, 8B, 10B
Fertile soil	3C, 5C, 6C
Barren soil	1E
Estuarine mud	1F, 2F, 3F
Lagoon system*	1G, 2G, 3G, 4G, 5G
Laboratory isolates	1H, 2H, 3H
Biological filter*	1I, 2I
Activated sludge*	1J, 2J
Biological filter	1K, 2K
Activated sludge*	1L
Minewater (pH 7)	1M

* These systems were treating carbonization liquors.

Planning the tests. Since at least 100 characters were required for the numerical analysis, some difficulty was experienced in devising a sufficient number of tests, and at the same time avoiding bias in any particular aspect. To overcome this the tests were designed to fall into four groups; substrate tests including carbon and nitrogen sources, physiological tests such as growth at selected temperatures and pH values, inhibition tests with both organic and inorganic compounds, and certain tests on organic media in view of the presence of certain facultative autotrophic strains.

For many of the tests a common substrate was required which could easily be measured to assess the amount of growth which had occurred. The only suitable energy source utilized by all the strains described in this paper was thiosulphate. In the preliminary experiments with this substrate it was found that even under the most rigidly standardized conditions many of the strains exhibited variable rates of thiosulphate oxidation when tested on different occasions. This variability was decreased as the test period was extended to 28 days, and this rather long test period was, therefore, used throughout the investigation. In many of the experiments this variability was further decreased by expressing the results as a percentage of the thiosulphate oxidized in a control series which was run alongside the test under 'standard conditions'.

The final selection of the tests was made on strictly logical grounds and only those were rejected which were either all positive or all negative or those which were obvious duplicates.

Tests. The basal mineral salts medium, S0, was (g./l. distilled water): Na_2HPO_4, 1·2; KH_2PO_4, 1·8; $MgSO_4$, 0·1; $(NH_4)_2SO_4$, 0·1; $CaCl_2$, 0·03; $FeCl_3.6H_2O$, 0·02; $MnSO_4.4H_2O$, 0·02. The S6 medium was prepared by adding $Na_2S_2O_3.5H_2O$, 10 g., to this basal medium and S7 by the addition of NH_4CNS, 0·02 g. The more

acidic S5 contained KH_2PO_4, 2 g. as the only phosphate, and NaCl, 1 g. to correct the ionic balance. The other energy sources tested were added to the S0 medium in the amounts given in Table 3. Where possible AR chemicals were used.

Table 3. *Tests*

A. *Substrates and carbon and nitrogen sources*

Test compound	Concentration (%)	Comments	Characters
Sulphur	Excess	Sterilized by steaming on 3 successive days	2
NH_4 thiocyanate	0·02	Analytical determination using a colorimetric test	2
Hydrogen sulphide	—	See text	2
No carbon source	—	Carried out in restricted atmosphere using doubly distilled water and appropriate guard tube to prevent access of carbon dioxide or ammonia. Scored against control series, see text	3
Sodium carbonate	0·05		3
Glucose	0·02		3
No nitrogen source	—		4
K nitrate	0·02		3
K nitrite	0·02		3
Na glutamate	0·52		2
Nutrient media	—	Growth on plate	2
Thioacetamide	0·02	Titrated with iodine	3
S7 plate	—	Criterion: sulphur deposition	2

B. *Physiological tests*

Medium	Variable	Comments	Characters
S6	19°	Rate of thiosulphate oxidation	3
S6	29°	Rate of thiosulphate oxidation	3
S6	35°	Amount of thiosulphate oxidation	4
Modified S6	0·5 % thiosulphate	Amount of thiosulphate oxidation	3
	1·0 % thiosulphate		3
	2·0 % thiosulphate		3
	4·0 % thiosulphate		3
	6·0 % thiosulphate		2
S5	pH		2
S6	None	Criteria: amount and nature of sulphur deposition	4
S6	None	pH consistently attained in this medium	5
S6 + nutrient	None	Amount of thiosulphate oxidation	3
S6 plate	None	Criterion sulphur: deposition	2

C. *Inhibition tests*

Inhibitor	Concentration (%)	Comments	Characters
Ampicillin	—	These tests were done on S6 plates using 'Sentest' tablets. (Evans Medical Ltd.)	2
Bacitracin	—		2
Celbenin	—		2
Chloramphenicol	—		2
Novobiocin	—		2
Streptomycin	—		2
Mixed phosphate	4	The results were scored as a percentage of the amount of thiosulphate oxidized relative to a parallel control series grown under the standard conditions	4
Sodium chloride	5		2
Potassium nitrate	1		3
Ammonium chloride	2·5		3
NH_4 thiocyanate	200 p.p.m.		2
Phenol	25 p.p.m.		4
Phenol	100 p.p.m.		4
		Number of occasions the final pH was alkaline rather than acid for the autotrophs in the above inhibition tests	3

Table 3 (*cont.*)

D. *Heterotrophic tests*

Gelatin	Liquefaction of charcoal gelatin discs, Oxoid	2
Nutrient broth	Putrefactive odour	2
Kosers citrate	Oxoid	2
Starch	Hydrolysis	2
Phenol	Utilization of 200 p.p.m. phenol	2
Tallow agar	Lipolysis	2
Tributyrin agar	Lipolysis Oxoid	3
Glucose peptone	Acid to phenol red	2
Nitrate peptone	Gas and nitrite formation	4
S6/1 % glutamate	Growth on plate, autotrophic or heterotrophic	2
S6/1 % glutamate	Inhibition of thiosulphate oxidation	2
S6/1 % glutamate	Yellow fluorescence	2

The inoculum used in the tests was 0·02 ml. of a 3-day-old culture grown under the standard conditions. The standard conditions, also used for the control cultures, were the S6 medium, 10 ml., in a 50 ml. conical flask at 28°.

Analysis. In the majority of tests, the result was obtained by titration of one ml. of the thiosulphate medium with 0·01 M-iodine. The final pH value of the tests was recorded using a Pye Universal pH meter with a glass electrode. The change in pH value was used as a criterion of growth in the sulphur and hydrogen sulphide tests.

Method of scoring tests. The results were scored according to the method of Beers & Lockhart (1962), and the S values calculated according to the method given by Sneath (1957).

RESULTS

The strains were arranged in order of similarity and the result is shown in Table 4. Four groups were clearly differentiated corresponding to *Thiobacillus trautweinii*, *T. novellus*, *T. thioparus* and *T. neapolitanus*. These have been numbered 0, 1, 3 and 4; group 2, comprising the anaerobic species will be described in a later paper. In addition to the two authentic strains of *T. trautweinii*, group 0 includes organisms which are very similar to other named species. Thus 5C is very similar to *Pseudomonas stutzeri* and 1F has been tentatively identified as *P. fluorescens*. These organisms are characterized by their ability to grow as facultative autotrophs, oxidizing thiosulphate to polythionates with a corresponding increase in the pH value of the medium.

Only three strains are present in group 1, two authentic strains of *Thiobacillus novellus*, and one new isolate (1L). The latter strain was lost shortly after being tested, and was thus one out of only three organisms which could not be maintained indefinitely. These organisms grow in both organic and thiosulphate media, and in the latter case decrease the pH value.

Of the 20 strains in group 3, ten are new isolates, four are authentic cultures of *Thiobacillus thioparus*, three are authentic cultures of *T. thiocyanoxidans* and three unnamed strains. The organisms of this group oxidize thiocyanate, and the final pH in thiosulphate media does not fall below 3·5. Although slight differences were found between the cultures received as *T. thioparus* and *T. thiocyanoxidans*, they were not sufficient to warrant a further subdivision of this group.

Group 4 is the most acid tolerant of the four species, the pH in thiosulphate media

Table 4. *Sneath diagram*

Group 0

1	3F	00
2	1F	94 00
3	2B	75 80 00
4	1A	75 80 93 00
5	5C	74 73 69 75 00
6	p4	71 71 71 73 80 00
7	d1	69 67 73 77 74 91 00
8	3C	63 64 74 76 66 76 80 00
9	d2	69 64 72 75 64 75 78 80 00
10	10B	69 69 70 72 63 66 70 78 84 00
11	4B	65 64 65 67 63 62 67 72 80 96 00
12	h4	64 52 62 61 63 65 70 70 79 81 79 00
13	t3	63 63 65 60 57 62 62 62 73 72 71 62 00

Group 1

14	a1	46 45 57 56 52 54 56 70 59 58 57 55 73 00
15	b2	49 48 56 56 53 56 58 69 61 59 57 55 73 96 00
16	1L	46 47 55 48 46 43 43 63 48 61 53 51 66 85 81 00

Group 3

17	2K	43 42 33 28 31 30 30 38 35 42 35 35 49 51 51 59 00
18	1K	45 43 34 29 33 32 32 39 34 41 34 33 46 54 55 62 91 00
19	1B	37 38 42 42 33 32 34 46 42 37 44 35 54 63 64 63 83 72 00
20	2F	40 38 37 37 34 34 39 46 44 43 42 43 54 63 64 68 88 79 86 00
21	1H	34 32 37 34 32 27 32 42 34 41 37 38 46 59 62 68 76 88 81 86 00
22	2H	34 38 44 37 32 29 32 49 34 43 39 40 49 63 64 73 73 78 83 88 91 00
23	6C	40 38 39 34 32 32 37 42 39 41 37 40 51 59 64 67 76 79 81 91 91 93 00
24	1E	40 35 46 39 32 29 34 46 37 41 37 43 49 63 64 73 71 74 81 86 88 93 91 00
25	2G	34 38 46 39 29 27 29 46 32 43 37 38 46 61 62 75 71 74 81 86 88 98 91 95 00
26	t1	34 32 39 32 30 27 32 44 34 41 34 38 49 61 57 70 83 86 76 83 88 86 83 83 83 00
27	3B	35 33 38 32 32 24 32 38 30 36 35 36 43 54 55 69 78 82 74 82 84 84 84 87 87 90 00
28	p2	40 38 34 34 38 32 42 41 39 43 44 43 49 61 62 65 83 86 81 88 81 81 83 79 79 86 90 00
29	p1	40 38 34 34 38 32 42 39 37 41 44 40 46 56 60 55 73 76 74 83 76 79 81 76 76 79 87 91 00
30	h3	34 32 32 34 33 28 32 42 34 39 39 39 44 59 61 62 76 79 79 83 88 83 83 81 81 81 82 88 83 00
31	h2	29 30 29 32 35 24 32 39 29 38 42 38 39 54 57 58 76 79 74 81 86 83 81 79 81 81 82 88 86 93 00
32	p3	26 27 29 27 30 20 27 32 24 35 39 30 39 45 45 53 71 74 71 69 81 74 76 69 71 79 82 79 81 83 86 00
33	b5	30 31 30 33 36 25 33 38 30 39 45 38 41 51 51 54 66 68 73 73 73 73 76 68 71 73 81 83 83 83 83 90 00
34	h1	29 30 29 32 35 24 32 37 29 38 44 35 39 49 50 52 63 67 74 76 74 76 79 71 74 71 79 81 86 83 86 91 98 00
35	b5	24 24 27 27 24 15 24 27 21 33 30 27 38 44 44 50 59 62 74 71 71 74 77 74 74 68 80 77 82 79 83 91 97 00 00
36	a7	26 27 26 29 33 24 32 32 24 30 37 28 28 39 41 43 61 64 64 67 69 67 69 62 64 67 68 71 76 73 81 86 88 91 91 00

Group 4

37	8B	29 30 34 37 29 22 27 27 22 27 37 25 33 46 45 42 51 53 60 45 52 48 43 50 50 40 53 50 52 53 55 55 51 50 56 50 00
38	7B	28 31 28 26 26 18 21 28 15 31 31 24 32 46 43 55 62 63 48 43 53 48 43 45 50 50 53 53 45 53 55 58 49 48 50 48 75 00
39	1G	35 33 35 33 30 23 28 35 25 39 43 31 40 48 49 49 45 46 44 37 42 42 37 44 44 39 51 44 51 44 44 54 48 49 52 44 71 78 00
40	5G	26 19 32 27 27 22 33 27 23 24 29 28 28 37 38 43 51 52 41 43 52 43 43 50 45 50 61 48 52 44 50 55 44 45 50 50 60 60 73 00
41	m1	26 27 24 27 22 15 20 20 15 19 24 18 18 22 21 25 44 43 36 29 38 33 29 36 36 38 40 36 38 38 41 48 39 38 44 45 67 60 66 69 00
42	2J	29 24 24 24 30 20 24 27 20 24 32 23 28 37 38 38 46 48 45 41 48 43 41 43 41 43 50 52 50 49 50 60 56 57 59 62 64 58 68 76 83 00
43	p5	26 22 20 22 23 10 15 20 12 19 24 18 23 29 29 43 51 50 41 36 45 38 36 43 41 45 50 43 45 49 50 57 49 48 53 50 67 65 71 74 88 76 00
44	4G	24 22 20 22 20 12 17 15 12 14 20 15 15 22 21 25 37 36 29 24 33 26 24 31 29 29 37 29 33 34 36 45 42 41 47 48 57 55 83 74 84 74 81 00
45	2I	21 19 24 20 20 14 19 20 14 24 27 20 28 36 35 43 49 48 41 41 48 43 41 43 45 45 50 45 48 51 50 57 56 55 59 55 64 73 69 76 84 83 79 71 00
46	3G	18 16 24 20 17 10 15 17 10 19 24 18 21 29 29 33 39 31 36 31 38 33 31 38 36 33 45 36 41 42 41 50 49 48 56 48 60 63 73 76 71 86 81 83 88 00
47	1I	30 28 30 30 28 15 20 23 20 22 28 23 24 30 29 31 43 42 34 29 39 32 29 37 34 37 43 37 42 43 44 54 45 44 46 49 59 63 68 71 83 76 81 85 78 81 00
48	6B	26 19 30 30 27 23 33 30 23 27 33 31 28 40 39 45 43 42 45 39 48 42 39 48 45 39 44 42 42 47 45 52 47 45 52 48 68 73 77 74 84 77 81 84 87 85 90 00
49	1M	25 19 26 26 26 19 26 26 19 22 29 27 23 36 34 43 45 44 44 38 47 41 38 47 44 38 46 41 41 45 44 50 45 44 50 44 75 77 84 75 84 84 84 84 94 91 94 94 00
50	3H	37 37 21 26 21 11 16 21 16 26 26 16 33 45 45 44 55 55 40 40 40 40 40 40 40 45 40 45 45 50 50 50 55 55 58 60 60 74 75 60 65 77 85 75 90 80 65 90 90 00
51	b1	24 19 17 20 20 10 15 17 10 16 22 15 21 32 31 35 44 43 38 33 41 36 33 41 38 33 42 38 38 44 43 48 46 45 53 48 64 68 73 71 74 86 88 83 86 91 76 87 94 95 00
52	c1	24 19 18 20 21 10 15 18 10 17 23 15 21 32 32 36 44 44 39 34 42 37 34 42 38 34 43 39 39 44 44 49 46 46 53 49 63 67 90 73 73 88 88 81 85 90 75 87 94 95 00 00
53	c2	24 19 15 17 23 12 17 20 12 16 24 18 23 34 33 33 46 45 41 36 43 38 36 38 36 36 40 41 41 46 45 50 49 48 53 50 62 65 88 69 72 88 86 81 83 88 73 84 91 95 98 98 00
54	1J	32 27 24 27 28 15 20 22 15 22 27 20 23 34 33 38 46 45 41 36 43 38 36 43 41 36 45 41 41 46 45 50 49 48 53 50 67 68 71 61 71 88 81 81 83 83 78 87 94 85 93 93 91 00

falling to 2·8. The only other outstanding feature of this group is the resistance to inhibitors, particularly inorganic salts such as sodium chloride and ammonium chloride. Three of the 18 strains are authentic cultures of *Thiobacillus neapolitanus*, and the remainder new isolates.

The ten S values from the comparison of duplicated strains range from 88 to 100, with a mean value of 95·7. These figures include both the differences which may have arisen between the strains and the errors of testing. An indication of the error involved in testing is given by the S value of 97 between b5 and b5a which are the same strain divided immediately prior to testing.

Considerable doubt has been expressed about the validity of *Thiobacillus neapolitanus* (Starkey, 1956; Vishniac & Santer, 1957; Vishniac & Trudinger, 1962) and six further sets of S values were calculated to ensure that the differentiation between this species and *T. thioparus* was not caused by the particular set of tests selected. For this purpose 21 strains were statistically selected and S values calculated using the tests in sections A, B and C. Similar calculations were then made using these tests arbitrarily split into two, and the tests divided into three on the basis of the original design, substrates, physiology, and inhibition.

Inspection of the resulting six Sneath diagrams indicated that a separation between the groups corresponding to *Thiobacillus thioparus* and *T. neapolitanus* had been achieved in all cases. However, a more objective assessment of the data was required which would correct for the small population sample on which the study was based. Groups may be compared in terms of an imaginary central organism, but it was thought that the selection of a real central strain was closer to bacteriological practice as this allows comparative testing for the purposes of identification. The central organism was obtained by calculating the mean and standard deviation of the S values of each strain of the *T. thioparus* group, to the other members of the group, and selecting the central strain which had the smallest standard deviation. This procedure gives the strain which is most typical of the group as a whole. From the mean and standard deviation of the S values of the central strain it is possible to construct a probability curve as shown in Fig. 1. This gives the probable distribution of the S values of the *T. thioparus* group if an infinitely large number of strains had been examined. The mean and standard deviation of the *T. neapolitanus* strains to the central organism of the *T. thioparus* group were then calculated and the curve drawn on the same axes.

DISCUSSION

Despite the fairly wide application of multivariate analysis to bacterial taxonomy very few tests of the validity of the method have been reported. The results given in this paper fully support the concept that the classification of bacteria by overall similarity is independent of the tests used, and suggest that the number of tests required to give an adequate classification may have been overestimated (Sokal & Sneath, 1963). These conclusions, however, only apply to thiobacilli and in view of the unusually well-defined groups found within this genus should not be extended to other bacterial groups without verification.

The very well-defined species and the absence of intermediate forms in this genus found here are unusual as compared with similar studies which have been made

of heterotrophic organisms (Sokal & Sneath, 1963). The most probable explanation of this is that classification in other groups has been carried much further, and that one of the Thiobacillus species would be regarded in other groups as a genus, or an even higher taxonomic rank. Alternatively, the rather extreme

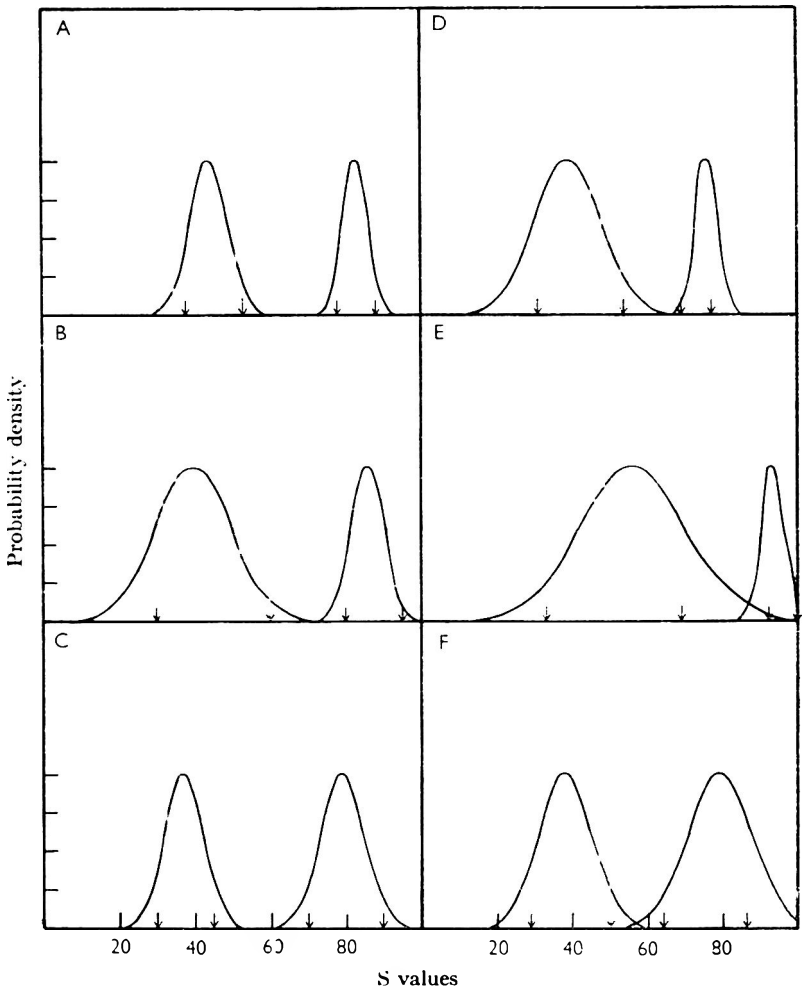

Fig. 1. The distribution of S values for *Thiobacillus thioparus* and *T. neapolitanus*. Group 3, *T. thioparus*, and group 4, *T. neapolitanus*. A: complete set of tests, 111 characters; B: split tests (i), 55 characters; C: split tests (ii), 56 characters; D: physiological tests, 40 characters; E: substrate tests, 34 characters; F: inhibition tests, 37 characters. The arrows indicate the numerical limits of each group.

environment occupied by these organisms may exert a relatively high selection pressure and the genetic constitution which corresponds to an intermediate type may not be viable.

Starkey (1935*b*) and Baalsrud (1954) were of the opinion that *Thiobacillus trautweinii* should be excluded from the genus *Thiobacillus* on the grounds that the oxidation of thiosulphate to tetrathionate did not release sufficient energy for these organisms to live autotrophically. Although the present results support the exclusion

of this group from the genus *Thiobacillus*, some of the test results suggest that inability to fix CO_2 may be an alternative criterion for this division. The tests, however, were not designed to yield specific information on this point, and further detailed studies would be required before this concept could be established.

Independently of the criterion used, there appears to be little doubt that *Thiobacillus trautweinii* should be excluded from the thiobacilli, particularly when other strains within this group belong to other named genera. It is unfortunate in this respect that the evidence from numerical analysis does not assist in making this decision. Although the division between the different groups is well marked, there does not appear to be obvious inter-group division at which to separate the thiobacilli from the facultative heterotrophs.

The results for *Thiobacillus thioparus* and *T. neapolitanus* fully support the work of Parker & Prisk (1953), and there now appears to be little doubt about the validity of these two species. Observations on their behaviour in thiosulphate media indicate that the differences between them may be sufficiently large to suggest differing metabolic pathways. This might account for some of the present differences of opinion regarding the thiosulphate metabolism of these organisms, as in some cases no distinction has been made between these two species.

The evidence from the S values of the duplicated authentic strains indicates that these organisms are relatively stable over long periods of time, even when subjected to varying conditions of culture. In the authors' opinion the amount of variation which was found can be almost wholly attributed to differences which are found in these organisms when tested on different occasions.

The inclusion of the duplicate strains, particularly those of *Thiobacillus thioparus*, gave the impression of a subgroup within the *T. thioparus* group on the Sneath diagram. This was obviously an artifact, but does raise the far more general problem about the frequency of organisms with very high S values in numerical analysis, and whether one should eliminate such strains before inspecting the data for subgroups.

After careful consideration of the original literature the authors have concluded that the most appropriate names for the species described are those given in this paper. The major decision which led to this conclusion was that Beijerinck's original papers (1904*a*, *b*) do not give sufficient detail to identify the aerobic species which he described. The first adequate description was given by Starkey (1935*a*, *b*) who believed his organism was the same as that of Beijerinck and therefore called it *Thiobacillus thioparus*. This strain is still available in the various national collections and its characteristics correspond with the original description. The evidence given in this paper has shown that *T. neapolitanus* is distinct from *T. thioparus*, and that the name given by Parker & Prisk (1953) is therefore valid. The validity of *T. novellus* does not appear to have been questioned in the literature.

A diagnostic table for the identification of these species will be given in a later paper.

The authors wish to thank Professor A. L. Roberts for his continued support of this programme, and the British Coke Research Association for financial assistance. We are grateful to the research workers who supplied the cultures given in Table 1, and Dr K. S. Zinnemann for translating the papers of Beijerinck.

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Efflux of Macromolecules from Washed *Dictyostelium discoideum*

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SUMMARY

Dilution of washed *Dictyostelium discoideum* amoebae in distilled water leads to an essentially immediate and specific efflux of RNA and protein into the extracellular environment. A substance's ability to prevent leakage correlates with its ability to stimulate the rate of morphogenesis.

INTRODUCTION

As a result of studying the mechanism by which imidazole compounds affect the rate of morphogenesis in *Dictyostelium discoideum*, it was postulated that the site of action was at the cell surface or outside the amoebae (Krichevsky & Love, 1964*a*). One possibility by which such a postulated site could control the rate of development is through controlling the passage of materials across the cell membrane. Thus, it might be expected that compounds which stimulated the rate of morphogenesis could inhibit the leakage of intracellular components into the medium. The present report describes the ability of various stimulatory materials to decrease the efflux of specific macromolecular constituents from washed amoebae.

METHODS

The procedures for growing and harvesting the myxamoebae were described previously (Krichevsky & Wright, 1963). For each incubation mixture, the amoebae from one cookie sheet (15 × 10 in.) of solid growth medium were harvested just before complete ingestion of bacterial growth, washed free from residual *Escherichia coli* and finally suspended in distilled water to a final volume of 5 ml. Each incubation mixture had 4·8 ml. of the suspended amoebae in 48 ml. final volume. The incubations were done in standard 1 l. beakers on a six-place magnetic stirrer so that all suspensions were stirred at identical rates at ambient temperature (about 22°).

Cell-free filtrates of the incubation mixtures were obtained by vacuum filtration of 2 ml. samples using Millipore filters of pore size 0·45 μ and 47 mm. diameter (Millipore Filter Corporation, Bedford, Mass.; catalogue number HAWPO 4700)

Pentose-containing materials were assayed by the orcinol reaction of Mejbaum as modified by Horecker, Smyrniotis & Klenow (1953). Protein was determined by the method of Sutherland, Cori, Haynes & Olsen (1949). The ninhydrin assay

for amino acids was done by the procedure of Rosen (1957). Diphenylamine reacting materials were determined as described by Dische (1955). Nucleic acids were isolated by the procedure of Szybalska & Szybalski (1962) except that the cell-free filtrates were not treated with sodium lauryl sulphate before NaCl addition.

RESULTS

Preliminary experiments had indicated that assaying cell-free filtrates from the suspensions for their content of ultraviolet-absorbing materials provided a convenient method for testing the postulate that stimulants of development could

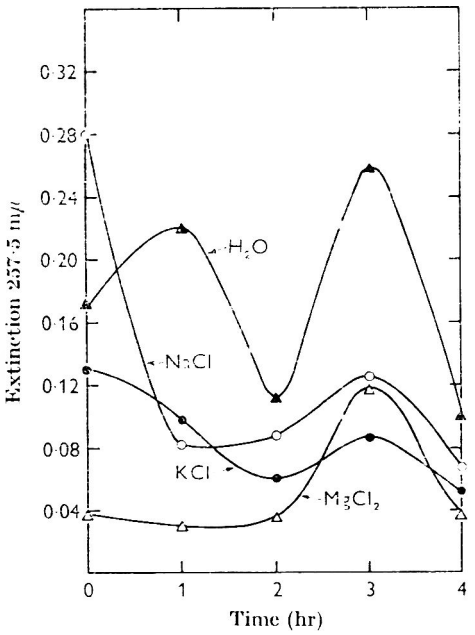

Fig. 1

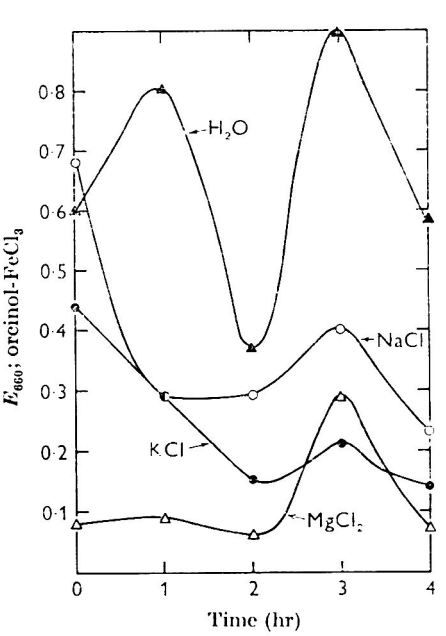

Fig. 2

Fig. 1. The leakage of u.v.-absorbing materials in the presence of chloride salts. Amoebae were incubated in water (▲–▲); 0·01 M-NaCl (○–○); 0·01 M-KCl (●–●); or 0·005 M-$MgCl_2$ (× – ×). With 0·5 M-NaOH, a 1/20 dilution, of the cell-free filtrate was made of the incubation mixtures noted above at 0, 1, 2, 3, 4 hr. The extinction of a 1 cm thick sample was determined at 257·5 mμ.

Fig. 2. The leakage of pentose-containing materials in the presence of chloride salts. Amoebae were incubated in water (▲–▲); 0·01 M-NaCl (○–○); 0·1 M-KCl (●–●); or 0·005 M-$MgCl_2$ (× – ×). To 0·5 ml. samples of cell-free filtrates of each incubation mixture were added 0·5 ml. of 6% perchloric acid. The samples were centrifuged and the supernatant separated and retained. Both the precipitates and supernatant solutions were assayed for total pentose. Only the results of the precipitate assays are presented in this figure.

control leakage from the organisms. An example of the data is illustrated in Fig. 1. The u.v.-absorbing material found in the cellular environment was, for the most part, greatest in the case of the medium containing only water, next was 0·01 M-NaCl, then 0·01 M-KCl, and least was 0·005 M-$MgCl_2$. The most striking departure from this ranking may be seen in the 'zero time' samples for water and NaCl. Since

the amoebae were added to the suspending medium and the suspension was filtered, the earliest samples obtained are not true initial samples but of the order of 2- to 4-minutes samples. Therefore, the release of the u.v.-absorbing material must have occurred within that period.

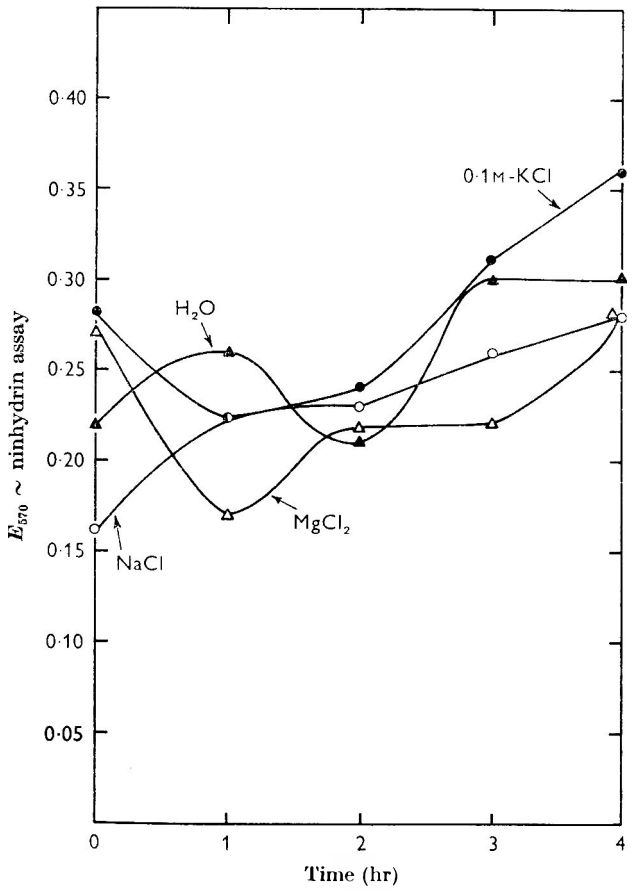

Fig. 3. The leakage of ninhydrin-reactive materials in the presence of chloride salts. Amoebae were incubated in water (▲–▲); 0·01 M-NaCl (○–○); 0·01 M-KCl (●–●); or 0·005 M-$MgCl_2$ (× – ×). To 0, 1, 2, 3 and 4 hr samples of cell-free filtrates of the above incubation mixtures were added equal volumes of 0·5 M-NaOH. One-tenth ml. of the diluted samples were assayed for their content of ninhydrin-reactive material.

The spectrum of various samples exhibited an absorption maximum at about 260 mμ. In preliminary experiments, the material appeared to be precipitable by cold perchloric acid. Therefore, the samples described in Fig. 1 were tested for their pentose content see Fig. 2. The qualitative relationship between the u.v.-absorbing material in the cell-free filtrate as a whole and in the perchloric acid-precipitable, pentose-containing material was consistently close (Fig. 1 *vs.* Fig. 2). Thus, in the experiments to be described below, the u.v.-absorption data were always obtained but will be omitted with the understanding that they were qualitatively the same as the pentose assay data.

When the perchloric acid-soluble fractions from the experiment shown in Figs. 1 and 2 were assayed for pentose content, it was found that there was little or no significant difference with either time or treatment. Furthermore, the values were all low, i.e. on the order of E0·04 to 0·06 in most of the cases (the range was 0·02 to 0·09).

To ascertain whether the release of materials into the medium was a generalized phenomenon, the experiment in Fig. 3 was performed. In this case, the cell-free filtrates were assayed for their content of ninhydrin-positive material. It may be seen that these materials were released in an entirely different manner than the pentose-reacting material. No differences in amounts of the ninhydring-reacting material could be attributed to the nature of the suspending medium.

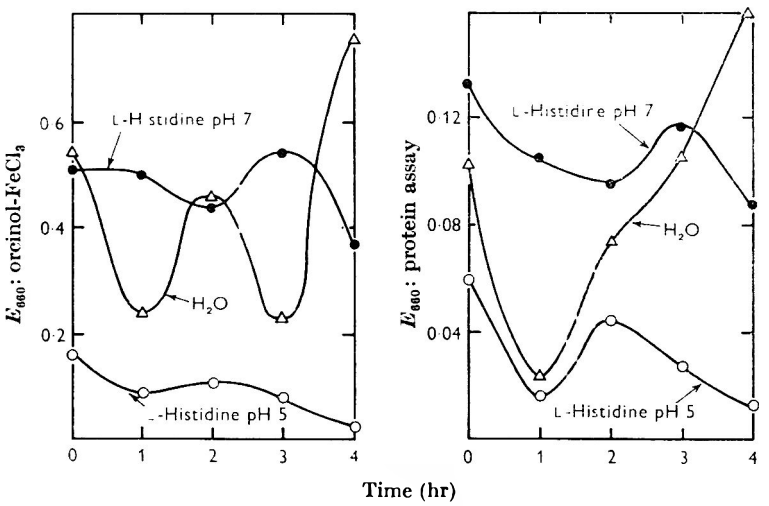

Fig. 4. The pentose and protein contents of cell-free filtrates in the presence of L-histidine. Amoebae were incubated in water (△–△); 0·04 M-L-histidine, pH 5 (○–○); or L-histidine, pH 7 (●–●). Samples were taken for pentose determinations as described for Fig. 2. Instead of the final centrifugation, the samples were filtered on 25 mm Millipore filters (type HA). The precipitates were redissolved in 1 ml. of 0·25 M-NaOH and assayed as usual (shown on the left).

The same procedure was used to obtain the samples for protein assay, except that the perchlorate-treated samples were placed in a boiling water bath for 15 min., cooled, filtered and redissolved in 0·3 ml. 0·5 M-NaOH. The results of the protein assays are shown on the right.

The effect of L-histidine on the leakage of macromolecules from washed amoebae is illustrated in Fig. 4. When supplied at pH 5, L-histidine markedly decreased the amount of perchloric acid precipitable material found in the cell-free filtrate as compared to either distilled water or L-histidine supplied at pH 7. The foregoing result was qualitatively the same whether the assay procedure used was for pentose or protein material. However, the kinetics and the quantitative relationships were found to differ in detail.

Figure 5 depicts the results of substituting D-histidine for the L-isomer. As in the previous experiment, D-histidine at pH 5 was more effective in preventing the efflux of macromolecular constituents from the amoebae than was either water or D-histidine at pH 7.

The effects due to sodium phosphate buffers were complex (Fig. 6). Initially, pH 7 sodium phosphate buffer was incapable of preventing the leakage of pentose-containing material as compared with distilled water. When the pH of the phos-

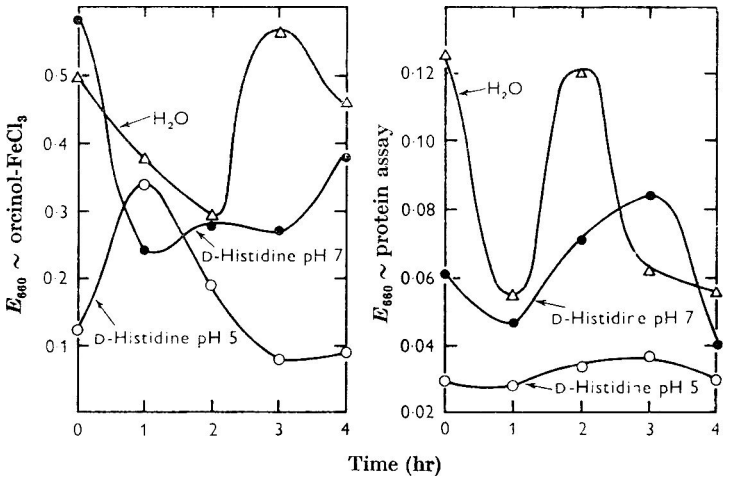

Fig. 5. The pentose and protein leakage from cells incubated in D-histidine. All conditions were the same as those described for Fig. 4 except that D-histidine was used in place of L-histidine.

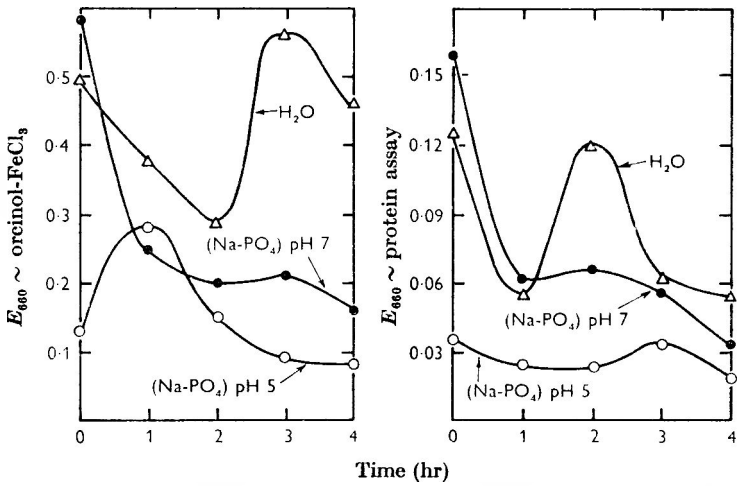

Fig. 6. The pentose and protein leakage from cells incubated in (sodium) phosphate buffer. All conditions were the same as those described for Fig. 4 except that 0·01 M (sodium) phosphate buffers were substituted for the L-histidine.

phate was 5·0 there was a marked lowering of extracellular pentose-containing material found throughout the sample period. It may be seen that the effect of the hydrogen ion concentration of the suspending medium was less important with the passage of time. In contrast, the pH of the sodium phosphate medium exerted a greater effect on protein leakage. Since the buffer was adjusted to a constant

phosphate concentration, the effects noted above may have been due to the increased sodium concentration as opposed to any pH effect (e.g. see Fig. 2).

In order to assess the significance of the leakage of the materials to the total system, the data for the incubation of amoebae in distilled water from four separate experiments were combined as shown in Fig. 7. It may be seen that as much as

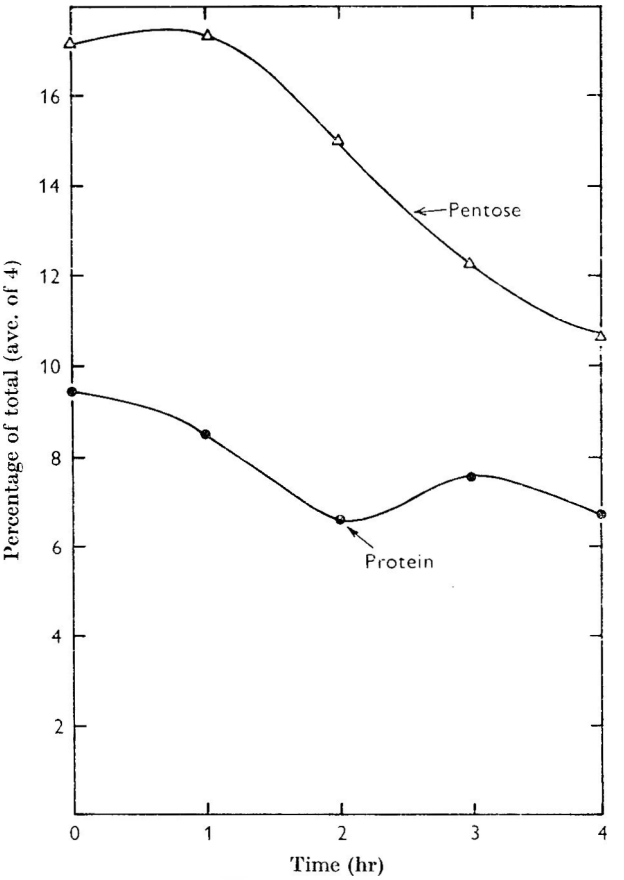

Fig. 7. The proportions of macromolecular leakage from amoebae incubated in distilled water. The data shown in this figure are the averages of four different experiments performed as described in Fig. 4. The incubation medium was distilled water in all cases. The values for the total amount of pentose and protein reacting materials were obtained by taking a second initial sample and omitting the filtration step so that the cellular contents were included in the samples. The appropriate assays were performed on 1/10 dilutions.

17 % of the perchloric acid-insoluble pentose-reacting material contained in the amoebae leaked into the medium (the range of all samples being 6·4–26·4 %). In the case of protein the high average was also the earlier and was about 9·5 % (range 0·8–15·0 %). The likelihood of the extracellular amounts of the two types of macromolecules being correlated with time, was tested by determining the correlation coefficients of each of the four experiments separately and by pooling the data within the individual experiments and determining the over-all correlation coefficient. The

values found were: 0·96, 0·49, 0·63, and 0·97 for the individual experiments and 0·665 for the pooled data. In two out of the four experiments here considered the correlation coefficients were highly significant ($P \leqslant 0{\cdot}01$) though the number of observations was very small for each group. Furthermore, the pooled value indicates that the correlation of the two factors (i.e. extracellular amounts of protein and pentose-containing materials at the various times) is significant also ($P \leqslant 0{\cdot}01$).

The pentose-containing perchloric acid-precipitable material was partially characterized as follows: first, the yield of orcinol reactive material extruded from amoebae in suspension in water or 0·04 M-L-histidine (at pH 7), was compared with that obtained by perchloric acid-precipitation or ethanolic precipitation. In all samples the yields were approximately the same regardless of the method of sample preparation. Little or no diphenylamine reactive material was isolated by either of the above procedures even though the samples for assay were increased by a factor of five. In addition, the material isolated by ethanolic precipitation exhibited similar spectral properties to commercial yeast RNA, with a 260/280 mμ ratio of approximately 2·03 to 2·15 in various samples.

DISCUSSION

The data presented here indicate that when washed amoebae from the growing amoeboid stage of *Dictyostelium discoideum* are diluted in distilled water, there is an immediate and specific efflux of macromolecules from the amoebae into the medium. The types of macromolecules concerned are ribosenucleic acid and protein. Deoxyribonucleic acid (as diphenylamine reactive material) was not detected in the cell filtrates. Low molecular weight ninhydrin-positive materials leaked into the medium in the same way, irrespective of the nature of the medium.

The leakage phenomenon could not be ascribed to simple osmotic shock. This is indicated by the observation that compounds at approximately equivalent tonicities varied widely in their capacity to prevent the leakage. When the suspending fluid was L-histidine at pH 5·0, the efflux of RNA and protein was almost completely prevented. In contrast, when the L-histidine was adjusted to pH 7·0, the leakage was as great and often greater than that found when distilled water was the diluent. Another example is provided by the series NaCl, KCl, and $MgCl_2$. Although the ionic strength of the $MgCl_2$ solution was less than that of the other two salts, it was by far the most effective in preventing leakage.

Continuing incubation of cell suspensions after the initial efflux of macromolecules (in those permitting leakage) for the most part resulted in either an over-all smooth lowering of the extracellular amounts or, an oscillation of these. The oscillation consisted of an initial drop in level followed by a secondary rise. The changes in RNA and protein with the passage of time were probably not random and independent phenomena. This was indicated by the statistical analysis of the replicate incubations wherein water alone was the suspending medium. It may be speculated that the change in extracellular amounts of RNA and protein are under cellular control. If the changes were due to extracellular destruction (in the cases where lowering was observed) it seems unlikely that the amounts of the two types of macromolecules would be so well correlated.

The data presented here suggest that the ability of various materials to stimulate

the over-all rate of morphogenesis in *Dictyostelium discoideum* is correlated with their prevention of leakage of RNA and protein from the amoeboid cells under the test conditions. In the series of chloride salts tested, NaCl was only slightly stimulatory, KCl more so and $MgCl_2$ very stimulatory (Krichevsky & Wright, 1963). As shown in Figs. 1 and 2, the same ranking obtained with respect to the ability of these salts in preventing the efflux of RNA. At pH 5, L-histidine is both a stimulant of the rate of morphogenesis (Krichevsky & Wright, 1963; Krichevsky & Love, 1964*a*, *b*) and an inhibitor of macromolecular leakage; at pH 7, it is neither. Although not as striking, the results were similar with D-histidine and sodium phosphate buffers.

Thus, materials which stimulate the rate of differentiation seem indeed capable of action at the surface of the cell. It may be postulated that the RNA and protein which leak from the amoebae are not indispensable for cellular differentiation, but provide stimulatory intermediates through their catabolism to monomeric constituents, and this only when available inside the organism. Certainly, it is well documented (Wright, 1964) that endogenous RNA and protein are degraded during morphogenesis.

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The cell walls of streptococci

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SUMMARY

The cell-wall compositions of 197 strains of streptococci were determined by paper chromatography. The residues formed 26 patterns, but 117 strains had one or other of only 3 patterns. The results suggest possibilities for future investigations of some poorly defined taxa.

INTRODUCTION

The methods developed by Salton (1953) made possible the study of the cell-wall composition as a relatively simple procedure and recent work by others has suggested that cell-wall composition may be of use in classifying streptococci (Roberts & Stewart, 1961; Slade & Slamp, 1962). As part of a general study of the taxonomy of the streptococci we have collected over 300 strains representing the recognized Lancefield groups, other named species and a variety of presently unclassifiable strains. Each of the strains has been examined by a series of some 50 physiological and biochemical tests, the results of which will be presented separately; 197 of the strains have been subjected to cell-wall analysis and the results are presented here. The results obtained confirm previous work to the extent that the cell-wall structure of a few clearly defined species is constant, but the many different patterns of cell-wall residues in the less well-defined clusters of strains indicate that the study of cell-wall composition, as a method of classification, is subject to the same limitations as have long been recognized with other methods.

However, strains usually classified as, or with, *Streptococcus viridans* fell into two major divisions on the basis of cell-wall composition. The significance of this finding is not yet clear, but it does suggest possibilities for the investigation of the 'species' *S. viridans*, which is now defined in terms of the absence of distinguishing characters. The results also suggest that some, at least, of the non-haemolytic streptococci of Lancefield groups A, C and G could be classified, not with their respective groups, but with the non-haemolytic strains of group F and the proposed species *S. milleri* (Guthof, 1956).

METHODS

Strains. 103 of the strains were obtained from culture collections in this or in other countries, and 94 strains were freshly isolated. All cultures were preserved in the freeze-dried state.

Preparation of cell walls. The cocci were grown for 2 days in 0·5 l. amounts of Todd Hewitt broth, except for the strains of *Streptococcus pneumoniae* which were grown in a broth made up of pancreatic digest of casein (1 %, w/v), yeast extract, Difco

(0·5 %), glucose (0·5 %) and K_2HPO_4 (0·5 %) at pH 7·0. Cultures of *S. cremoris* and *S. pneumoniae* were incubated at 32°; all other cultures were incubated at 37°.

The cocci were deposited by centrifugation and washed twice by resuspending in cold physiological saline after centrifugation. The first cell-wall preparations of six strains, the pneumococci and one strain in each of the Lancefield groups D, R and S were lost during handling. This was thought to be due to autolytic enzymes and in repeat preparations the washed cocci were heated in a boiling water bath for 10 min. before further treatment.

The washed cocci were resuspended in cold physiological saline, one drop of iso-octanol added, and the suspension shaken in a Mickle tissue disintegrator with an equal volume of Ballotini grade twelve glass beads for 10 min. The cell walls were separated from the glass beads by passing the mixture through a small sintered glass filter, and then the walls and the few remaining intact bacteria were deposited by centrifugation for 10 min at 12,000*g*. The deposit was washed twice with molar saline and resuspended in M/15 phosphate buffer (pH 8·0) containing trypsin 1 mg./ml. The suspension was incubated overnight at 37° with a few drops of toluene.

The cell walls were then washed twice with distilled water and twice with M-NaCl. The material was resuspended in molar saline and a differential centrifugation done by spinning at 1000*g* for 15 min. to deposit intact cocci and debris, and subsequently spinning at 12,000*g* for 10 min. to deposit the cell walls from the supernatant fluid of the previous operation. Loss of cell-wall material at the stage of differential centrifugation was great. The cell walls were then washed thrice with distilled water, or until the supernatant fluid was free from chloride and the preparation was then freeze-dried. The yield of material from 0·5 l. of broth varied between 1 and 32 mg. depending on the strain.

The method of preparation of cell walls is a modification of that described by Salton & Horne (1951) and was suggested by Salton (personal communication).

Paper chromatography. Three chromatographic systems were used routinely for all cell walls, but on occasions further tests were made and these are described below. For each chromatogram the products of acid hydrolysis of 2 mg. of cell walls were repeatedly dried in a vacuum desiccator containing concentrated sulphuric acid and potassium hydroxide pellets and finally applied to Whatman grade 1 paper.

To detect hexoses and deoxyhexoses, the dried walls were hydrolysed with 2 N-HCl for 2 hr at 100°. The residues were separated on a descending chromatogram on which the solvent was allowed to run for 16–20 hr. The solvent was butanol + pyridine + water (6 + 4 + 3, v/v). The paper was then air-dried and dipped through *p*-anisidine hydrochloride in butanol (1 %, w/v), and heated in an oven at 70–75° for 15 min. The detecting agent, used in this way, did not react with amino sugars, amino acids or polyols.

For the detection of polyols, the cell walls were hydrolysed in 2 N-HCl for 2 hr at 100°. These conditions largely converted ribitol to anhydroribitol. A periodate and benzidine reagent (Smith, 1960) was used to detect glycerol and anhydroribitol; ribitol was rarely present on the chromatograms. Separation of glycerol and anhydroribitol was achieved with a descending chromatogram on which butanol + acetic acid + water (12 + 3 + 5, v/v) was allowed to run for 40 hr.

Amino acids and amino sugars were detected in samples hydrolysed for 16 hr in 4 N-HCl at 100°. An ascending two-dimensional technique with 10 in. square papers was used. Butanol + acetic acid + water was the first solvent, and lutidine + water (13 + 7, v/v) was used as the solvent in the second direction. Residues were detected by dipping the air-dried papers through a solution of ninhydrin in acetone (0·2 %, w/v) and heating the paper in an oven (70–75° for 15 min.) These chromatograms were preserved by dipping them through an aqueous half-molar solution of nickel sulphate and then drying the papers in the oven.

Reproducibility of results. To test the reproducibility of the results reported below, fresh preparations of cell walls were made from 26 strains. The strains were not chosen at random, for with 7 of the strains the residue glycerol had previously been reported and anhydroribitol had been recorded as present in the residues of 12 strains. The same sugars, amino sugars and amino acids were recorded on the first and second occasions, but glycerol and anhydroribitol were each recorded on one less occasion in the second series of tests. This suggests that glycerol and anhydroribitol are indicative of polymers either less constantly formed or less firmly attached to the cell wall.

Grouping reactions. For the purposes of this paper we have taken the reaction of a strain with any Burroughs Wellcome Lancefield grouping antiserum of the groups A to S as indicating the presence of a group hapten. The cell wall composition is tabulated, where possible, by the presence of these haptens.

RESULTS

Table 1 lists the patterns of cell wall residues found and shows that, while twenty-six patterns of the occurrences of sugars and sugar-like substances were detected, more than half of the strains fell into only three of these patterns. Table 2 is a listing of these patterns against accepted or possible taxonomic groupings of streptococci and the final column of this table contains those strains in which the finding of further characters for differentiation would be useful. Table 3 gives the results obtained with individual strains from the National Collection of Type Cultures and the National Collection of Dairy Organisms.

The substances detected

Amino acids and amino sugars. The pair of solvents used to separate these compounds gave good separation of amino sugars, but had limitations for the separation of amino acids, which tended to lie diagonally across the paper. Aspartic acid and glutamic acid were only partly separated and thus, although aspartic acid was recorded as being present in sixty wall preparations, this may represent only a portion of the occurrences. Muramic acid, glucosamine, lysine, glutamic acid and alanine were detected in all cell walls. Valine and leucine (or isoleucine) were also present but in smaller amounts, and were absent from one strain (NCTC 6681). Glycine was found in small amounts in all but fifteen preparations, and serine was detected, as well as glycine, in the pneumococci and two other strains.

A residue with mobility between that of glucosamine and muramic acid in the solvent butanol + acetic acid + water was detected with ninhydrin in 11 strains. A positive Elson-Morgan reaction (Smith, 1960) indicated it was an amino sugar.

Table 1. *The patterns of occurrence of sugars and sugar-like substances in hydrolysates of streptococcal cell walls. Glucosamine and muramic acid were detected in all strains. The amino acids found are discussed in the text*

Cell wall pattern numbered	Residues making up the cell wall pattern									No. of strains found
	Rhamnose	Glucose	Galactose	6-Deoxy-talose	Galactos-amine	Fucosamine	Unknown amino sugar	Glycerol	Anhydro-ribitol	
1	+	−	−	−	−	−	−	−	−	4
2	+	−	−	−	+	−	−	−	−	5
3	+	−	+	−	+	−	−	−	−	6
4	+	+	−	−	+	−	−	−	−	8
5	+	+	−	−	−	−	−	−	−	10
6	+	+	+	−	−	−	−	−	−	47
7	+	−	+	−	−	−	−	−	−	2
8	+	+	+	−	−	−	−	−	+	1
9	+	+	+	−	+	−	−	−	+	9
10	+	+	+	−	+	−	−	+	−	6
11	+	+	+	−	+	−	−	−	−	46
12	+	−	+	+	−	−	−	−	−	1
13	+	+	−	−	−	+	−	−	−	1
14	+	+	+	−	−	−	+	−	−	1
15	+	+	+	−	−	+	−	−	−	8
16	−	+	+	−	+	+	−	−	−	1
17	−	+	+	−	+	−	+	−	−	1
18	Trace	+	+	−	+	+	−	−	+	1
19	−	−	+	−	−	−	−	−	+	1
20	−	−	+	−	+	−	−	−	+	3
21	−	+	+	−	+	−	−	+	−	3
22	−	+	+	−	−	−	−	−	−	1
23	Trace or −	+	+	−	−	−	−	−	+	4
24	Trace or −	+	+	−	+	−	−	−	+	24
25	Trace	+	+	−	+	−	−	−	−	2
26	−	−	−	−	−	−	−	+	−	1

Table 2. *Strains examined for cell wall composition divided by taxonomic group and cell-wall constituents. The letters of the alphabet refer to Lancefield group reactions, and the numbers in the body of the table are numbers of strains*

Cell wall pattern	A	C	G	B	D	Q	R	S	T	L	CO_2 dependent	F	Ottens types	*S. milleri*	Indifferent A, C and G	N	E	*S. uberis*	F	H	*S. sanguis* (gp. H)	*S. sanguis*	K	*S. salivarius* (gp. K)	*S. salivarius*	M	O	Pneumococci	Strains lacking distinguishing characters
1	3	.	.	.	.	.	.	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
2	.	5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
3	.	.	5	.	.	.	.	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
4	.	.	.	.	.	1	.	.	.	.	.	3	.	.	2	2	.	.	.	.	.	.	.	.	.	.	.	.	.	.
5	.	.	.	.	.	.	.	.	.	.	.	1	.	1	1	.	.	1	.	1	1	.	.	.	1	.	.	.	.	3
6	.	.	.	7	.	.	2	1	.	.	2	.	3	4	1	2	3	.	.	1	4	2	3	2	3	.	.	.	.	7
7	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	.	.	.	.	.	.	.	1
8	.	.	.	.	.	.	.	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
9	.	.	.	.	6	.	.	.	.	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	1	.	.	.	1
10	.	.	.	.	6	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
11	.	.	.	.	3	.	.	1	2	1	.	15	4	3	3	2	1	.	.	.	2	3	.	3	.	.	.	.	3
12	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.
13	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.
14	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.
15	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	3	4	.	.	.	.	.	.	.	.	.	1
16	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1
17	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1
18	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1
19	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	.	.	.	.	.	.	.	.
20	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	2	.	.	.	.	.	.	.	1
21	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	3	.	.
22	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1
23	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	.	.	.	3
24	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	2	3	.	.	2	6	.	.	11
25	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	.	.	1
26	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1
No. of strains	3	5	5	7	15	1	2	2	2	4	2	20	7	8	7	7	6	1	3	6	7	11	6	5	4	4	7	3	37

Table 3. *The results of cell wall analysis of strains of streptococci obtained from the National Collection of Type Cultures and the National Collection of Dairy Organisms*

Strain no.	Species and serological group	Cell wall pattern no.
NCTC 8194	*S. pyogenes* gp. A	1
NCTC 8181	*S. agalactiae* gp. B	6
NCTC 4669	*S. dysgalactiae* gp. C	2
NCTC 7136	*S. equisimilis* gp. C	2
NCTC 6176	*S. zooepidemicus* gp. C	2
NCTC 7379	*S. faecium* gp. D	9
NCTC 8175	*S. faecalis* var. *liquefaciens* gp. D	10
NCTC 8176	*S. faecalis* var. *zymogenes* gp. D	11
NCTC 8177	*S. bovis* gp. D	11
NCTC 8213	*S. faecalis* var. *faecalis* gp. D	10
NCTC 8307	*S. durans* gp. D	11
NCTC 5385	*S. sp.* gp. E	13
NCTC 3858	*S. uberis* gp. E	6
NCTC 5389	*S. sp.* gp. F	11
NCTC 8037	*S. sp.* (MG) gp. F	11
NCTC 8547	*S. sp.* gp. G	3
NCTC 7868, NCTC 10231	*S. sp.* gp. H	6
NCTC 7863	*S. sanguis* gp. H	5
NCTC 7865	*S. sanguis* gp. H	6
NCTC 10232	*S. sp.* gp. K	24
NCTC 8606	*S. salivarius* gp. K	6
NCTC 8618	*S. salivarius* gp. K	11
NCTC 6404	*S. sp.* gp. L	11
NCTC 7760	*S. sp.* gp. M	9
NCTC 6681	*S. lactis* gp. N	4
NCDO 712	*S. lactis* gp. N	6
NCDO 176	*S. lactis* var. *diacetilactis* gp. N	4
NCDO 607	*S. cremoris* gp. N	11
NCDO 924	*S. cremoris* gp. N	6
NCTC 8031	*S. sp.* gp. O	24
NCTC 9824	*S. sp.* gp. P	15
NCTC 9938	*S. sp.* gp. Q	4
NCTC 10234	*S. sp.* gp. R	6
NCTC 7366	*S. salivarius*	6
NCTC 7864	*S. sanguis* type II	20
NCTC 7466	*S. pneumoniae*	21
NCTC 3165	*S. sp.* 'viridans' type	11

In the solvent butanol + pyridine + water the order of separation was the same as glucose and in the solvent phenol + aqueous ammonia (phenol 160 g., water 40 ml. in an ammoniacal atmosphere) the mobility of the unknown sugar was 2·0 relative to glucose (Crumpton, 1959). This unknown amino sugar was thus thought most likely to be fucosamine, and it showed identical mobilities with fucosamine obtained by extraction of *Chromobacterium lividum* (*C. violaceum*) NCTC 7917 (Crumpton & Davies, 1958) in these three solvent systems.

6-Deoxyhexoses. Rhamnose was present in considerable amounts in 155 strains and was recorded as being present in trace amounts in 10 further strains. Of these 10 strains, 7 would in everyday usage be classified as *Streptococcus viridans* and are included in the final column of Table 2, 2 were strains of *S. sanguis* and 1 belonged to Lancefield group 0. The possibility exists that if more sensitive methods had been

used for the detection of rhamnose, the proportion of strains recorded as showing smaller amounts of rhamnose would have been higher and the number of strains described as lacking rhamnose would have been correspondingly lower.

One strain (S320) received as *Streptococcus cremoris* but differing from typical strains of this species by its failure to yield an extract reacting with Lancefield group N serum gave a sugar travelling closer to the solvent front than rhamnose. This sugar was obtained in chromatographically pure form by elution from paper chromatograms. In the CyR10 reaction for 6-deoxyhexoses of Dische & Shettles (1948) samples of the unknown sugar gave spectra with the maximum value occurring between 396 and 400 mμ and a zero reading at 430 mμ; indicating a 6-deoxyhexose. The $R_{rhamnose}$ values were 1·09 in butanol + pyridine + water, and 1·20 in butanol + acetic acid + water. An authentic sample of 6-deoxy-L-talose showed the same mobility in the latter solvent, and similar colour reactions were given by both sugars with a vanillin + perchloric acid spray (MacLennan, Randall & Smith, 1959). Electrophoresis with constant current (0·1 mA./cm.) and a voltage range of 150 to 300 V. in borate buffer pH 9·5 showed identical mobilities of the unknown sugar and 6-deoxy-L-talose (MacLennan, Randall & Smith, 1961). The unknown sugar was therefore thought most likely to be 6-deoxytalose.

The strains examined

Lancefield group A (*Streptococcus pyogenes*). The cell-wall compositions of 26 strains of *S. pyogenes* (Lancefield group A) has been described by Michel & Gooder (1962). They found that rhamnose was the only neutral sugar present, a finding confirmed by our examination of 3 strains. *N*-acetylglucosamine is the determinant of the group A antigen (McCarty, 1958), but the conditions of hydrolysis used will de-acetylate such sugars and the detection of residues found in hydrolysates of cell walls would not distinguish between glucosamine as part of a cell wall polysaccharide and glucosamine as part of the mucopeptide. As mentioned previously glucosamine and muramic acid were detected in all cell walls.

Lancefield group C. Cell walls of 5 strains of group C were examined and galactosamine was the only residue additional to those found in strains of *Streptococcus pyogenes*. This sugar in the *N*-acetyl form is the determinant of the group C antigen (Krause & McCarty, 1962). All 5 strains were found to have the same cell wall composition but Slade & Slamp (1962) and Michel & Gooder (1962) have recorded glucose as an additional residue present in the cell walls of some group C strains. Three strains of group C were from the National Collection of Type Cultures and are listed in Table 3, and the two further strains were isolated from human clinical material.

Lancefield group B. Seven strains of *Streptococcus agalactiae* (Lancefield group B) were examined and, of the neutral sugars, all contained rhamnose, glucose and galactose and all lacked galactosamine. Five strains showed only trace amounts of glucose on the paper chromatograms. Strains with these three aldose sugars and lacking galactosamine form pattern 6 in Table 1. One strain (EW 57) gave a precipitate with Lancefield group R sera as well as a group B serum but has been included as a strain of *S. agalactiae* as it had the physiological and biochemical reactions of this species and was unlike strains of group R. Slade & Slamp (1962) and Wittner & Hayashi (1965) have both recorded galactosamine as an additional

constituent of the cell wall of group B streptococci. This cell wall composition in Table 1 is designated as pattern 11, which with pattern 6 comprises almost one half of the strains examined.

Lancefield group G. Curtis & Krause (1964) have presented evidence of an antigenic relationship between the cell wall polysaccharides of groups B and G streptococci. However, our studies showed differences in gross cell wall composition in that all 5 haemolytic strains of group G contained galactosamine and lacked glucose while none of the strains of group B contained galactosamine and all contained glucose.

Lancefield group D. Of the species forming Lancefield group D some wall hydrolysates contained glycerol, some anhydroribitol and some neither. This is the basis of the division of these strains into patterns 9, 10 and 11 in Table 1. Two strains (BS 347 and BS 361) have been included by us in this group, for, although grouping reactions failed, in all other respects they showed the reactions typical of *Streptococcus faecalis*. Six cultures were common to our study and that of Slade & Slamp (1962); they were the cultures from the National Collection of Type Cultures listed as group D in Table 3. The same sugars were detected in both studies.

Groups Q, R, S and T. A total of only 7 strains, from culture collections, were examined in the groups Q, R, S and T. The strains in groups R, S and T showed the residues of the two most frequently found patterns, those numbered 6 and 11 in the Tables 1 and 2. One strain in group S, s 1230, was unusual in that a large amount of glycine was found in the cell wall and this strain was also autolytic.

Group L. Analysis of cell wall preparations from four different cultures of group L streptococci showed a different composition for each strain. Two strains were probably isolated from animals, the other two were isolated from hospital patients.

CO_2 dependent streptococci. These two strains were isolated by Fitzgerald & Keyes (1960) and were capable of causing dental caries in the hamster. They differed from the 'minute' streptococci found in groups F and G (Deibel & Niven, 1955) in that they required carbon dioxide for growth on normal laboratory media, that they were non-haemolytic (indifferent) when grown on horse blood agar plates, and in their absence of a recognized group antigen. Both strains had the cell wall composition numbered 6 while the 'minute' streptococci of group F examined by us had the cell wall pattern numbered 11.

Group F and physiologically related streptococci. Streptococci of Lancefield group F, particularly the non-haemolytic strains, have many physiological characters in common with strains that lack a recognized group antigen but contain an Ottens antigen (Ottens & Winkler, 1962); with streptococci of the proposed species *Streptococcus milleri* (Guthof, 1956); and with the few non-haemolytic streptococci of groups A, C, and G that we have examined. The 42 strains thus grouped together show considerable similarity in cell wall composition. The compositions of the cell walls of the indifferent strains of groups A, C and G are closer to each other and to the strains of group F than to the haemolytic strains of groups A, C and G. The non-haemolytic streptococcus of group A mentioned in the paper of Michel & Gooder (1962) may be similar to the organisms included here.

One strain (FW 113, pattern 5), extracts of which gave a delayed precipitate with group F antisera was of interest, for its cell walls lacked detectable amounts of both galactose and galactosamine. Michel & Willers (1964) partially hydrolysed the

polysaccharide of a group F strain and obtained a disaccharide of glucose and galactosamine which was a most potent inhibitor of F/anti-F reactions. From the results of inhibition tests these authors thought glucose to be the major determinant of antigenicity and the finding of this strain provides further evidence for that postulate.

Group N. Six cultures, extracts of which gave a precipitate with group N serum, were studied. They belonged to the species *Streptococcus lactis*, *S. lactis* var. *diacetilactis*, and *S. cremoris*, but their species designation was not correlated with their wall composition. One strain sent to us as a typical representative of *S. cremoris* (s320, pattern 12) could not be identified by serological methods but it gave the physiological reactions expected of a strain of this species. It was this strain that contained the unusual sugar 6-deoxytalose, a sugar identified by MacLennan (1961) in *Actinomyces bovis*.

Group P. The three strains belonging to group P were notable in that they all contained fucosamine but lacked galactosamine. Fucosamine was reported by Wheat, Rollins & Leatherwood (1964) as a cell wall constituent of an organism identified as *Bacillus cereus*.

Group E. Six strains belonging to Lancefield group E were examined. One was a strain of *Streptococcus uberis*, two were haemolytic, and three strains showed greening on horse blood agar. One of the haemolytic strains (NCTC 5385, pattern 13) has been examined by Cummins & Harris (1956*a*), Michel & Gooder (1962), and Slade & Slamp (1962). All of these workers described mannose as present in the cell wall of this strain. Within the limits of our techniques we did not confirm this finding but did detect fucosamine. One of the greening strains (s322, pattern 14) contained an amino sugar we were unable to identify. The chromatographic mobility differed slightly from that of fucosamine.

Streptococcus uberis. One strain of *Streptococcus uberis* that did not belong to group E was examined, and it differed in composition from the strain of *S. uberis* of group E by the absence of galactose.

Group H and Streptococcus sanguis. A working definition of serological groups was given in the section on methods and this definition needs clarification here, for de Moor (1964) has pointed out that both group H and group K are serologically heterogeneous. With the sera we used to examine our collection, strain Challis (NCTC 7868) belonged to group H while the strain Perryer (F 90 A) examined by Slade & Slamp (1962) did not, although both strains were isolated and characterized by Professor R. Hare as group H.

Seven strains of *Streptococcus sanguis* serological group H were examined and the only unusual feature of the cell walls noted was that of the 4 strains with the cell wall pattern numbered 6 in Table 2, 3 strains contained glucose and galactose at the lowest limit of detection. Six strains were examined that belonged to group H and that failed to produce a polysaccharide on sucrose-containing media, and 4 of these strains contained small amounts of fucosamine (pattern 15).

Strains that are classified as *Streptococcus sanguis* but lack the group H hapten are of interest, for two general patterns of cell wall composition can be seen in Table 2. Some strains have the commonest patterns found among the streptococci, containing rhamnose, glucose and galactose and either containing galactosamine (pattern 11) or lacking it (pattern 6). With the exception of one strain, the other

strains of *S. sanguis* not of group H differ markedly. The residue anhydroribitol is to be found and the walls lack or contain only trace amounts of rhamnose. One of Washburn, White & Niven's (1946) strains of *S. sanguis* type II (NCTC 7864) shows the latter general pattern.

Group K and Streptococcus salivarius. With one exception, the strains of group K and the strains of *Streptococcus salivarius*, whether or not they contained the K antigen, showed one or other of three cell wall patterns. Eleven of the 15 strains were found to belong to the patterns 6 and 11 (Table 2). Three of the strains of group K that did not produce levan differed markedly in cell wall composition from the other 12 strains in that rhamnose was not present while anhydroribitol was detected.

Roberts & Stewart (1961) found rhamnose to be absent from a number of their strains of group K, but present in strains of *Streptococcus salivarius* that contained the K antigen. Our results thus confirm and extend their findings.

Groups M and O. Streptococci of group M are described as possessing rhamnose in the cell wall (Roberts & Stewart, 1961; Slade & Slamp, 1962). We found rhamnose in the only haemolytic strain we examined (NCTC 7760, pattern 9), while it was absent from the cell walls of the 3 greening strains isolated from man. In all strains, including the one with rhamnose, anhydroribitol was detected.

The group O serum used in the identification of our strains contained antibodies reacting with components of streptococci of more general occurrence than the 'O' antigen (Stewart, 1965). Thus a fuller serological analysis of the strains included in group O might lead to some being excluded. Of the total of 5 strains examined by Roberts & Stewart (1961) and Slade & Slamp (1962) none contained rhamnose. Six of our strains were found to lack rhamnose and to yield anhydroribitol (pattern 24) and the seventh strain was found to contain rhamnose in an amount at the lowest level of detection (pattern 25).

The pneumococci. Three pneumococcal strains were examined and our findings confirmed those of Roberts & Stewart (1961) in that we did not detect rhamnose but did detect both glucose and galactose (pattern 21). Of particular interest was the finding of large amounts of glycerol in the residues of hydrolysis. Glycerol was detected among the residues from a number of other cell walls but then always at the lowest level of detection.

Strains lacking positive distinguishing characters. The last column in Table 2 contains the results from 37 strains that lacked positive distinguishing characters. If these strains are divided on the basis of cell wall composition, one strain (pattern 26) is clearly unlike any other, for it had the residues usually found in the cell wall of a staphylococcus (Cummins & Harris, 1956*b*). The 16 strains included in the patterns 5 to 15 had the residues usually associated with streptococci, namely rhamnose, one or more additional neutral sugars and the amino acids common to nearly all strains. The 20 strains in patterns 16 to 25 lacked entirely, or contained only small amounts of, rhamnose. The 37 strains showed considerable diversity in physiological tests but the strains in patterns 23, 24 and 25 were physiologically similar to the strains included in Lancefield group O.

DISCUSSION

In this study the residues present in hydrolysates of entire cell walls have been investigated as part of a taxonomic survey of the genus *Streptococcus*. The results of previous investigations are confirmed to the extent that well-established species such as *Streptococcus pyogenes* and *S. agalactiae* differ from each other in their component cell wall sugars, but unfortunately these species are the exception and very few taxa are composed of strains showing one cell wall pattern.

The current classification of streptococci is based, first, on the possession of a Lancefield group antigen; second, in a few species on the basis of a single striking marker such as levan production (*Streptococcus salivarius*); and third, on a composite picture of physiological activities. The taxa defined by these different bases do not always coincide, as exemplified again with *S. salivarius* which may or may not be group K. Cell wall composition seems to constitute yet another dimension in the classification scheme. The production of a dextran-like polysaccharide from sucrose is the diagnostic feature of *S. sanguis*, the species is serologically heterogeneous, and in addition it shows 7 different patterns of cell wall composition.

At the same time there are some parts of the genus in which cell-wall composition may perhaps offer some help. Thus the rhamnose-deficient strains (patterns 23, 24 and 25) may have sufficient other characters in common to suggest that they form a significant cluster. Apart from the total pattern, cell-wall analyses may be of value when it can be shown that particular residues are associated with an antigen as, for example, glucose in the group F antigen.

It should also be possible to find methods for separating the various structural components of the cell wall and it is possible that this would provide a more comprehensible basis for a taxonomic classification, but much further work is needed before this is possible. Meanwhile the present results show that there is a sufficient variety in the cell wall composition of the different members of the genus to suggest it might be possible to find some more satisfactory grounds for defining *Streptococcus viridans*, hitherto defined on the lack of distinguishing characteristics of the strains concerned.

The residues found are presumably components of the various sorts of polymers found in cell walls and which have been discussed by Salton (1964). Glucosamine, muramic acid and the main amino acids are presumably components of the mucopeptide. The amino acids present in lesser amount could come from a trypsin resistant peptide that adheres to the cell wall, or the mucopeptide may be of more complex composition than is usually described. Cell wall polysaccharides whether they be 'group' or 'type' in nature could contain amino sugars and neutral sugars, and teichoic acids would be expected to contain a polyol, alanine and a sugar. Little is known about the lipids of bacterial cell walls, but perhaps the glycerol found in the cell walls of the pneumococci might be a component of the recently described glycolipids of Gram-positive bacteria (Brundish, Shaw & Baddiley, 1965).

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Self-heating of Hay and Grain in Dewar Flasks and the Development of Farmer's Lung Antigens

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With an Appendix on Taxonomy by J. Lacey

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SUMMARY

In moist hay allowed to self-heat aerobically in Dewar flasks, the pattern of temperature change with time was affected considerably by the type of hay and duration of storage, but there was a relationship between water content and maximum temperature reached. Below 29 % water content there was little heating or antigen production; in the critical range of 29–34 % water content, different lots of hay self-heated to different temperatures between 33 and 55° and varied widely in their content of farmer's lung hay antigen complex (FLH), the wetter hays usually producing the more antigen; all samples with 40 % water heated to *c.* 65° and produced FLH antigen, associated with the presence of *Thermopolyspora polyspora*. Progressively less antigen, especially in the lower regions of the flasks, was produced as water content increased from 47 to 68 %. Moist barley and oat grain also self-heated and produced FLH antigen, usually only in the middle of the grain mass, where *T. polyspora* was most abundant; the drier upper layers and the lower regions where excess water accumulated were free from the antigen.

INTRODUCTION

The self-heating of baled hay has been studied in relation to the microbial and biochemical changes during moulding (Gregory, Lacey, Festenstein & Skinner, 1963) and to the development of antigens of immunological significance in farmer's lung disease (Gregory *et al.* 1964). Pepys *et al.* (1963) attributed the presence of these antigens (FLH complex) to the growth of the thermophilic actinomycetes *Thermopolyspora polyspora* and *Micromonospora vulgaris*.

The first laboratory experiments on self-heating of hay were made by Miehe (1907) who used a central wire container filled with hay surrounded by similar containers insulated with cotton-wool. Dewar flasks were used first by Hildebrandt (1927) and James, Rettger & Thom (1928) and later by Miehe (1930).

Our experiments in Dewar flasks began as studies on the effects of water and aeration on the self-heating process, in attempts to reach high temperatures. Hay very rich in FLH antigen was produced so the effect of water on antigen production

was examined in more detail. The chopped hay used in the wide-mouthed Dewar flasks was easily mixed with water, so avoiding sampling errors caused by uneven water distribution, which is unavoidable in baled hay. A few experiments were made with moistened barley and oat grain.

METHODS

Two batches of good quality air-dry hay were used in most experiments; 1961 hay from a timothy grass and fescue ley ('SB') hay, Gregory *et al.* (1963), used from March 1962 to November 1963, came from a stack stored in a barn; samples were periodically chopped into 1–3 cm. lengths and stored in polythene bags or in sacks at outdoor temperatures. The water content was 16%, except for the last batch in November 1963, when it was 19%.

The second batch of hay was baled in 1962 from a perennial ryegrass + meadow grass ley; a bale was chopped in November 1963 and used until March 1964. The water content, also 16% at the start, fell to 9% after storage in sacks in a heated greenhouse. The other hays were from bales made in 1962 (Yorkshire Fog + Agrostis ley) and 1963 (timothy + meadow fescue + white clover ley), referred to as 1962′ and 1963 hay, respectively.

A weighed quantity of chopped hay (usually 500 g.) was moistened in a polythene bucket to the required water content, thoroughly mixed by hand and then transferred to a 4 l. Dewar flask. A 1 l. and a 10 l. Dewar flask were also occasionally used, and in one experiment a barrel lagged with glass fibre sheet was filled with 15 kg. hay that had been moistened and mixed on a concrete floor. Water content is given as percentage wet weight, and weights of hay given all refer to the weight before adding water.

In experiments with barley (14% water content), 2·5 kg. grain was used and water added to give a content of near 40%, but the water was not all absorbed and some accumulated at the bottom of the 4 l. Dewar flask. In one experiment, as also in the experiment with oats (12% water content), the grain was left to stand with the added water with occasional mixing, for 5 hr, before all the water was taken up.

A plug of cotton-wool about 2 cm. thick was placed on the surface of the hay or grain and one or two extra similar layers placed on top and overlapping the sides of the flask to lessen heat loss. A thermometer was inserted into the centre of the mass and the 4 l. flasks contained a second thermometer 2–3 cm. from the bottom of the mass 25 cm. deep. The thermometer in the barrel was in a metal tube filled with paraffin oil, to ensure good contact with the bulb, and also to diminish the temperature change, when it was raised by thread to be read.

Grain removed at the end of the experiments was subdivided into layers according to the visual appearance of microbial growth. The hay was occasionally subdivided in this way, but usually into three portions of approximately equal weight, designated upper, middle and lower layers; only the middle layer was usually examined microbiologically.

The samples were analysed as described by Gregory *et al.* (1963) and Gregory *et al.* (1964), except that the short chopped hay and grain to be examined in the wind tunnel was placed in muslin bags to prevent too much of the material being blown away. For immunological work the hay and grain samples were extracted with

phenol saline after defatting, and the extracts Seitz filtered, dialysed and freeze dried. All extracts were tested for precipitin reactions in agar gel against a set of twenty sera, using the double-diffusion method of Ouchterlony, and by immuno-electrophoresis, as described by Pepys *et al.* (1963) and Gregory *et al.* (1964).

RESULTS

Factors affecting self-heating

Quantity of hay. Figure 1 shows that 50 g. hay at 40 % water content in a 1 l. Dewar flask self-heated to only 28°, but 250 g. attained 49°. Larger quantities of hay did not become much hotter, 350 and 500 g. self-heating to 54° and 58°, respectively; 15 kg. in the lagged barrel also reached 58°. The self-heating patterns were very similar, with broad maxima and no subsidiary peaks.

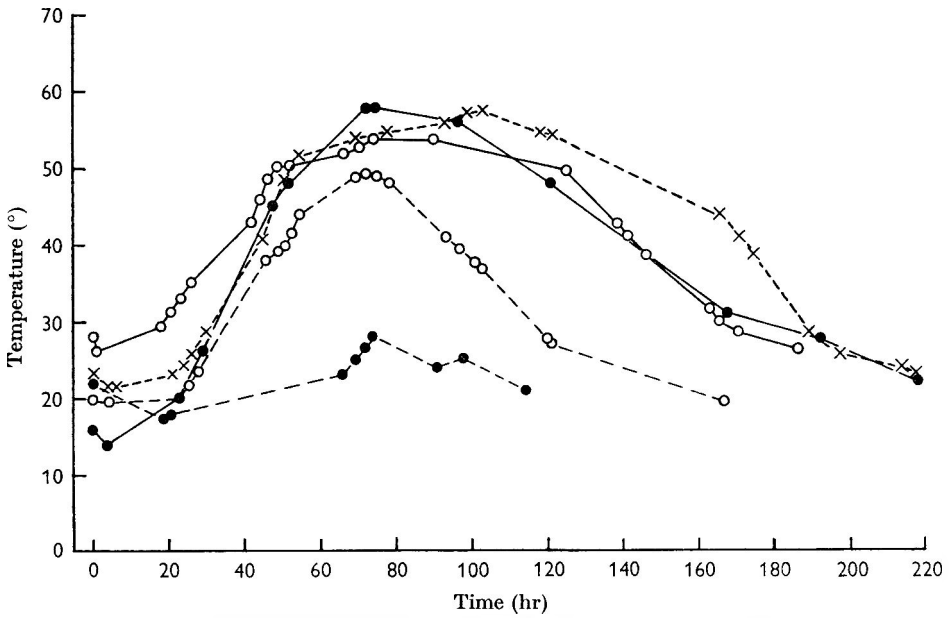

Fig. 1. Progress of self-heating of different quantities of 1961 hay, moistened to 40 % water content, (March 1962–January 1963). --●--, 50 g.; --○--, 250 g.; —○—, 350 g.; --×--, 500 g.; —●—, 15 kg.

Aeration. Aeration was usually by diffusion through the cotton-wool plug at the top of the flask, but in some experiments it was increased by passing air down a tube to the bottom of the flask. The flow rate was measured with a rotameter.

Additional aeration at 25 c.c./min. accelerated the cooling of 350 g. hay after the maximum temperature was reached, but had no effect on the self-heating pattern of 500 g. hay. Figure 2 shows the effect of varying the rate of airflow in a flask with 500 g. hay where entry of air by diffusion was prevented by closing with a wooden lid: the temperature rose to only 44° on aerating at 25 c.c./min., fell to 32° when aeration was stopped, and rose to 57° on starting it again at 40 c.c./min.

Aeration of 1250 g. hay in a 10 l. Dewar flask by diffusion through a cotton-wool plug only, was inadequate for maximum self-heating of the hay towards the bottom

of the flask. Maximum temperatures of 63°, 52° and 50° were attained in the upper, middle and lower parts, respectively; with additional aeration at 50 c.c./min., a maximum temperature of 68° was obtained at all levels. Temperatures found at the bottom of 4 l. flasks closed with cotton-wool plugs were usually a few degrees higher than those in the middle.

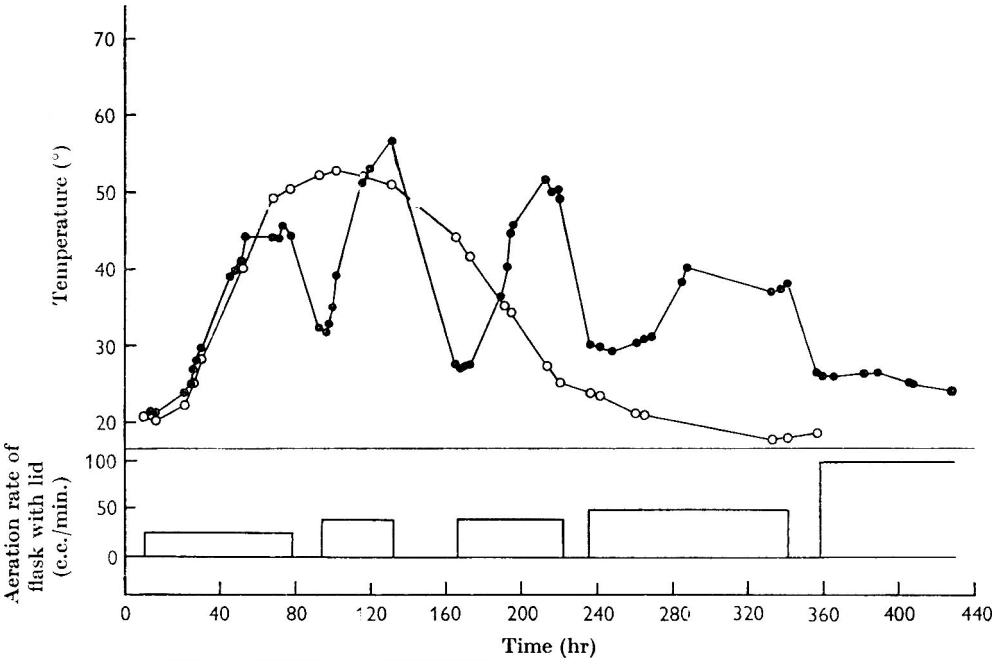

Fig. 2. Effect of aeration on self-heating of 1961 hay (500 g. moistened to 40 % water content; February 1963) ●, Dewar flask with fitting wooden lid aerated as shown; ○; flask with cotton wool plug, aerated by diffusion.

Water content, storage and type of hay. Figure 1 shows the self-heating pattern of 1961 hay in March 1962–January 1963, when there was one broad maximum only. Figure 3*a* shows that in September–October 1963 secondary peaks developed and these were particularly prominent in November 1963, especially at the larger water contents (Fig. 3*b*). Figure 3*c* shows heating patterns for 1962 hay, studied at different water contents, with mostly broad, single peaks as for the earlier 1961 hay. Two other hays examined at 40 % water content showed considerable differences, particularly in the times taken, 45 and 130 hr, respectively, to reach the maximum temperature (Fig. 3*d*). Increasing water content accentuated the heating pattern of any particular hay (Figs. 3*b*, *c*), hays with 30 % or less water content did not heat above 30°, hays with 57 and 68 % water content cooled slowly. Despite the differences in heating pattern, there is a general relationship between the maximum temperatures reached by the different hays and their water contents (Fig. 8).

Microflora in hays of different water content

Fungi. The total numbers of fungus spores released from the hay samples varied considerably (Fig. 4*a*) and peak concentrations reflected the abundance of different

species. Spores were abundant in hays of 28 to 29, 31, 34 and 47 % water content. The relative abundance of Aspergillus species gave a good guide to the initial water content of the hay. *Aspergillus glaucus* was most abundant in hay at 26 % water

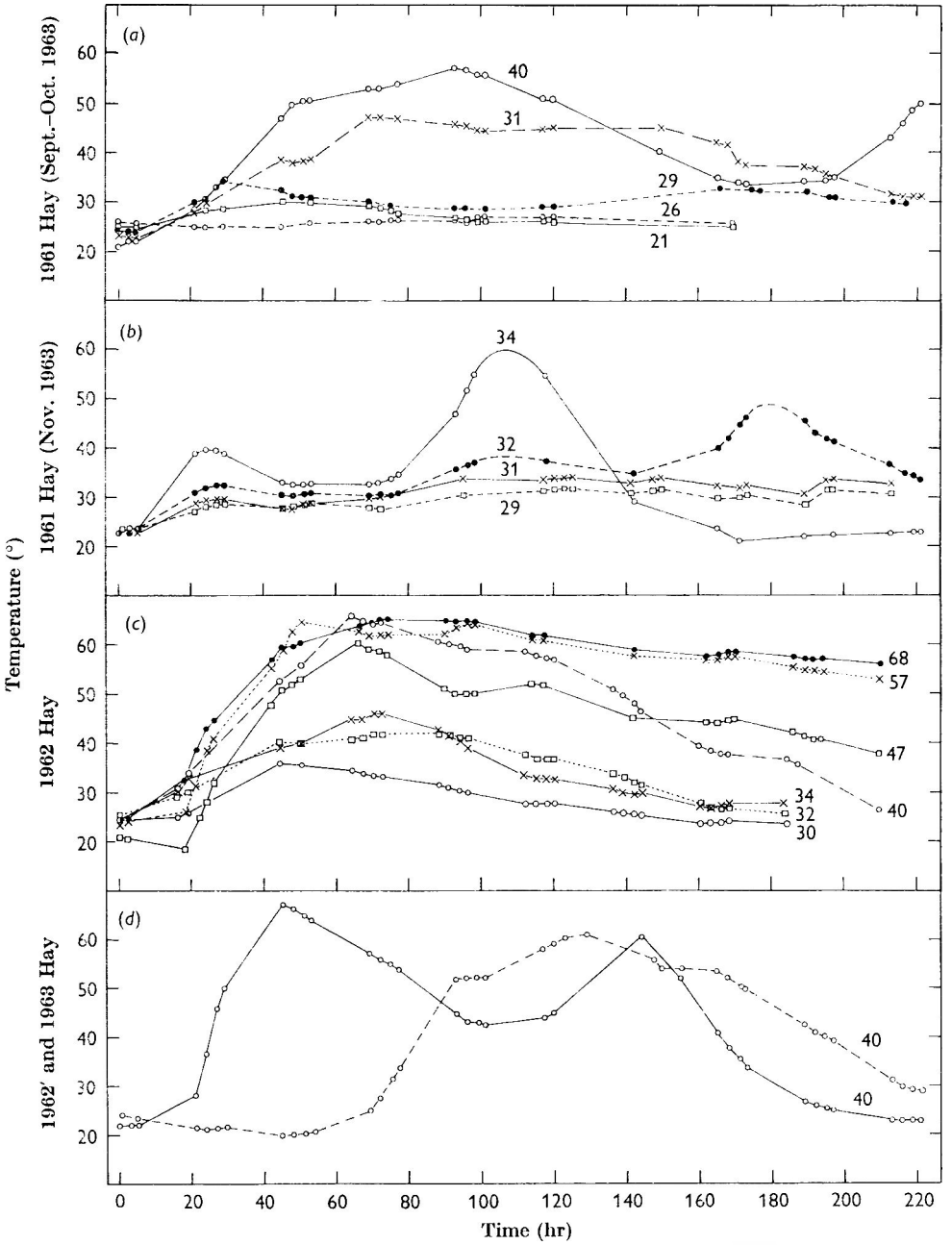

Fig. 3. Self-heating patterns of 500 g. quantities of different hays moistened to different water contents, also showing the effects of storage of 1961 hay (*a*, *b*); (*c*) 1962 hay; (*d*) ----1962' hay; —— 1963 hay. Figures on curves show water contents.

content (Figs. 4*b*, 5). *A. versicolor* and *A. nidulans* accounted for the peaks of Aspergillus type spores at 28–29 and 31 % water content, respectively, and *A. fumigatus* was most abundant at 40 % water content. Mucor type spores, probably mostly *Absidia* sp. were most abundant in hay at 34 % water content (Fig. 4*b*) and *Humicola lanuginosa* and *Paecilomyces varioti* caused the peak at 47 % on Fig. 4.

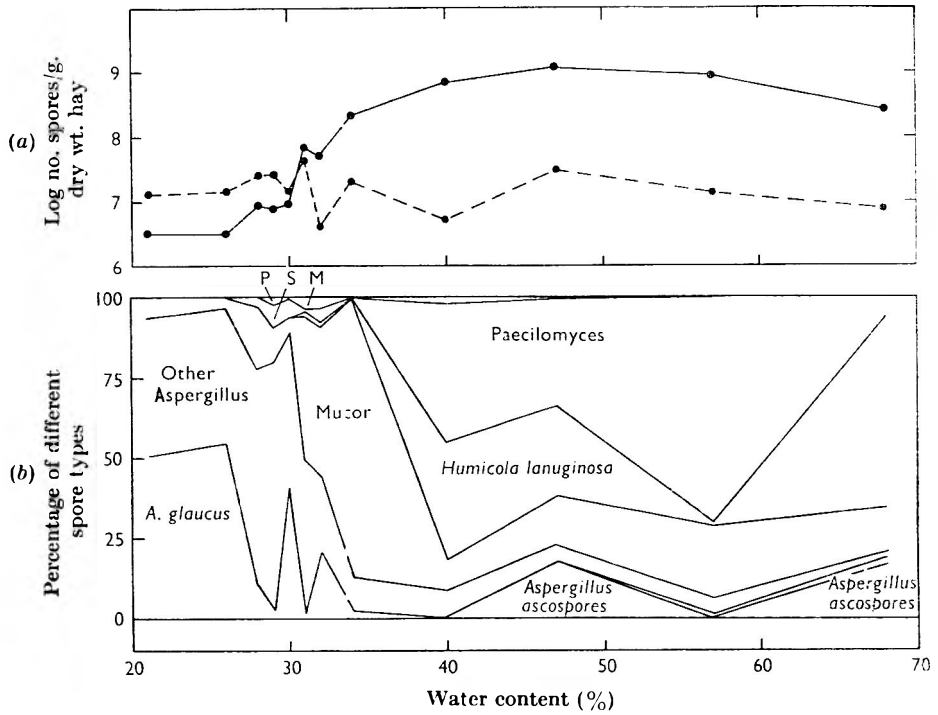

Fig. 4. Microflora in hays of different water content as determined by cascade impactor. (*a*), ---, fungi, ——, actinomycetes and bacteria; (*b*), proportions of fungal spore types. P = Paecilomyces, S = Scopulariopsis, M = miscellaneous.

Other species isolated included *Scopulariopsis brevicaulis* (most abundant at 28–29 % water content), *Mucor pusillus* (40–47 % water content) and occasionally *Malbranchea pulchella* var. *sulfurea*, *Aspergillus flavus*, *A. niger*, *A. terreus*, *Penicillium* spp., *Cladosporium* sp., and *Sporotrichum* spp. The values for the hay of 68 % water content are those in the upper layer, which had to be partly dried before wind tunnel examination and this may account for the apparent increases in spore numbers.

Actinomycetes and bacteria. Actinomycete spores and bacteria, as determined by the cascade impactor, were few in hay containing less than 30 % water (Fig. 4*a*); the hays of 40 % water content contained a mean of 430 million spores/g. dry wt. The most, 1220 million spores/g. dry wt. were at 47 % water content.

Bacteria growing at 25° and 60° were isolated in largest numbers from hay at 47 % water content, and those growing at 40° from hays of 34 % and 68 % water content. Up to 85 % of 40° bacteria from hays of 40 % water content were filamentous colonies of the *Bacillus licheniformis* type.

Andersen sampler plates incubated at 24° and 40° showed *Streptomyces fradiae* to be most abundant in hays at 31 % water content (Fig. 6). Few colonies of *Micromonospora vulgaris* or *Thermopolyspora polyspora* were isolated from hays at less than 32 % water content; most were isolated from hays at 68 and 47 %, respectively. *Thermopolyspora glauca* occurred in a similar way to *T. polyspora*.

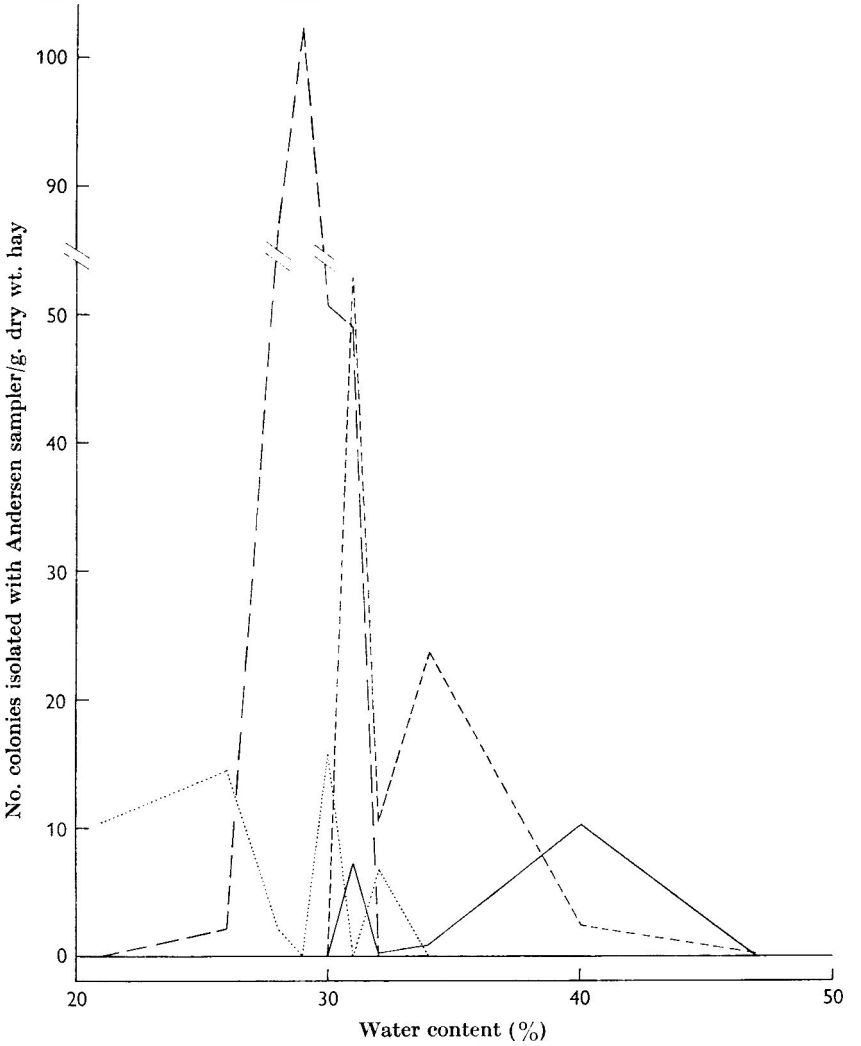

Fig. 5. Occurrence of Aspergillus species in hays at different water contents. —, *A. fumigatus*, — —; *A. versicolor*; ----, *A. nidulans*;....., *A. glaucus*.

FLH antigen

The early experiments with different quantities of hay at 40 % water content showed that extracts of the samples of 250 g. and 15 kg. hay reacted in the double diffusion test with 17 and 19, respectively, of 20 sera tested. Extracts of samples of the upper and lower halves of the 350 g. hay reacted with 8 and 19 sera, respectively; the upper layer contained predominantly fungi, especially *Aspergillus* spp.

and *Humicola lanuginosa*, and the lower layer contained these plus many white actinomycetes, which were also observed in the other samples mentioned above.

Effect of water content. Table 1 shows results for 500 g. quantities of hay of water content of 40% and less. The results are for the middle layers of the flasks only: extracts of the lower layer usually reacted with more sera than did those of the middle layer; extracts of the upper layer reacted with fewer sera, as with the 350 g. sample above.

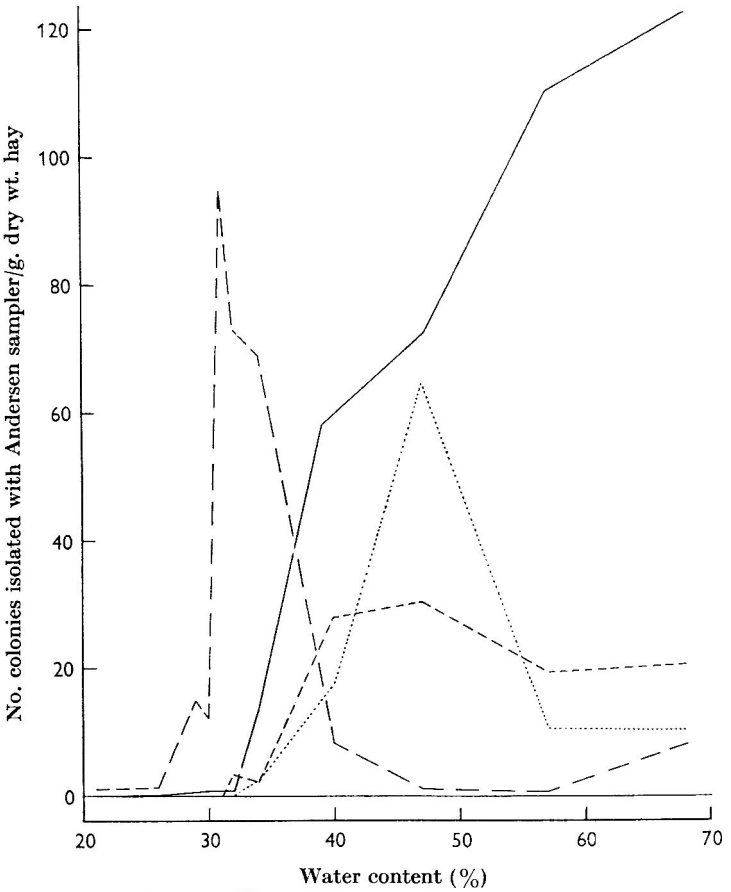

Fig. 6. Occurrence of actinomycete species in hays at different water contents. —, *Micromonospora vulgaris*; — —, *Streptomyces fradiae*; ----, *Thermopolyspora polyspora*; ·····, *Thermopolyspora glauca*.

The hay samples with 40% water content reached maximum temperatures of 57–66°. Extracts of these samples reacted with 18–20 sera and gave typical immunoelectrophoretic patterns (Pepys *et al.* 1963). Extracts of some hays in the range of 29–34% water content also gave typical patterns and these hays contained many *Thermopolyspora polyspora*, isolated by the Andersen sampler.

The hays of 29–34% water content self-heated to 33–55° and differed considerably in their content of FLH antigen. Those hays with less than 29% water self-heated little and their extracts reacted with few sera in the double-diffusion test.

Only occasional reactions were observed in the immuno-electrophoresis test and these consisted solely of the C line, which is typical of reactions given by fungi, particularly Mucor and by the actinomycete *Micromonospora vulgaris* (Jenkins, 1964). Very few *Thermopolyspora polyspora* were isolated from these samples.

Table 1. *Occurrence of FLH antigen in self-heated hay, classified according to immuno-electrophoretic analysis*

All experiments with 500 g. hay (initial weight). Results for middle layers of Dewar flasks.

Hay reference 1 = 1961 hay (for *a*, *b*, see Fig. 3) 2 = 1962 hay	Water content (%)	Max. temp. (°)	Number reacting sera (max. 20)	Number *T. polyspora* colonies/g. dry wt. hay	pH
		A, B and C lines			
		(i) Strong pattern			
1*a*	40	57	18	31·1	7·0
2	40	66	18	6·0	8·0
1962′ hay	40	61	20	—	7·2
1963 hay	40	67	19	47·2	7·7
1*b*	34	55	19	3·9	8·5
1*b*	32	47	17	6·3	7·6
		(ii) Weak pattern			
2	34	46	11	0·9	7·4
1*b*	31	34	14	0	6·9
1*b*	30	35	14	0	6·9
1*b*	29	32	10	0	6·9
		C lines only			
2	32	42	11	0·4	7·8
1*a*	31	47	6	0	7·3
1*a*	29	35	6	0	7·1
1*a*	28	36	6	0	7·3
		No lines			
1*b*	30	33	7	0·9	7·1
2	30	36	6	0·5	7·5
1*a*	26	31	5	0	7·3
1*a*	21	27	5	0	6·9

Table 2 shows the results for the wetter hays of 47–68 % water content, which self-heated to 61–65°. The more water the hay contained, the less FLH antigen was present, particularly in the lower part of the flask. *Thermopolyspora polyspora*, however, was abundant in all such hays, including the upper layer of the wettest hay, an extract of which reacted with only 8 sera and gave a weak immuno-electrophoretic pattern.

Microfloral succession and development of FLH antigen in hay of 40 % water content

Sequential events during self-heating were studied by filling each of eight flasks with 500 g. 1962 hay and emptying one flask every day. The flasks differed slightly in heating pattern, probably because of differences in packing, but the general

pattern was similar and the series can be regarded as sequential samples in the same experiment (Fig. 7).

Fungi. Fungal spores increased rapidly during the first 2 days when yeasts were predominant (Fig. 7). After 4 days numbers had declined to fewer than 1 million spores/g. dry wt. hay. They remained few until the 9th day, when numbers of *Humicola lanuginosa*, Paecilomyces, Mucor and Aspergillus type spores all increased.

Table 2. *Occurrence of FLH antigen in self-heated 1962 hay of water content between 47 and 68 %*

Water content (%)	Layer of Dewar flask	pH	No. reacting sera (max. 20)	No. *T. polyspora* colonies/g. dry wt. hay	Immuno-electro-phoretic pattern
47	Upper	7·5	18		Strong
	Middle	7·3	18	30	Strong
	Lower	6·7	19		Weak
57	Upper	6·8	18		Weak
	Middle	6·8	16	19	Very weak
	Lower	6·1	1		Negative
68	Upper	7·6	8	20	Weak
	Middle	7·8	4		C lines only
	Lower	7·1	1		Negative

Bacteria. Dilution-plate counts showed that bacteria able to grow at 25° increased from about 1×10^6 to $3{\cdot}7 \times 10^9$ organisms/g. dry wt. hay during the first day (Fig. 7). The colonies were almost all punctiform, buff or orange-yellow and consisted of Gram-positive cocci referrable to Micrococcus, Subgroup 6 (Baird-Parker, 1962). For the first 5 days this type of microflora remained essentially unchanged; although the numbers of bacteria increased still further; thereafter, the numbers declined but the proportion forming filamentous colonies, mostly *Bacillus licheniformis*, increased. After 6 days, filamentous colonies constituted over 68 % of the total, falling to 44 % on the 8th day, when the total numbers had declined to $1{\cdot}5 \times 10^8$/g. dry wt. hay.

On plates incubated at 40°, these filamentous colonies formed a significant proportion of colonies from most samples, though colonies of micrococci were also numerous. Numbers of filamentous colonies changed little throughout the experiment, but their proportion of the total increased up to the fifth day and then declined.

Thermophilic bacteria growing at 60° occurred only in the last three samples and formed 21 % of the total colonies in the last two; most colonies were thermophilic actinomycetes. The Andersen sampler detected a few thermophilic bacteria after 2 days and more from four days onwards.

Actinomycetes. Actinomycete spores and bacteria increased rapidly during the first 2 days, but, after a decline, numbers were still increasing at the end of the experiment (Fig. 7). Andersen-sampler plates indicated that most of the organisms during the early period were bacteria. Few *Micromonospora vulgaris* were isolated at 60° until the third day, but numbers rapidly increased to a maximum after 5 days. *Thermopolyspora polyspora* was first detected after 2 days at 60°, but colonies were

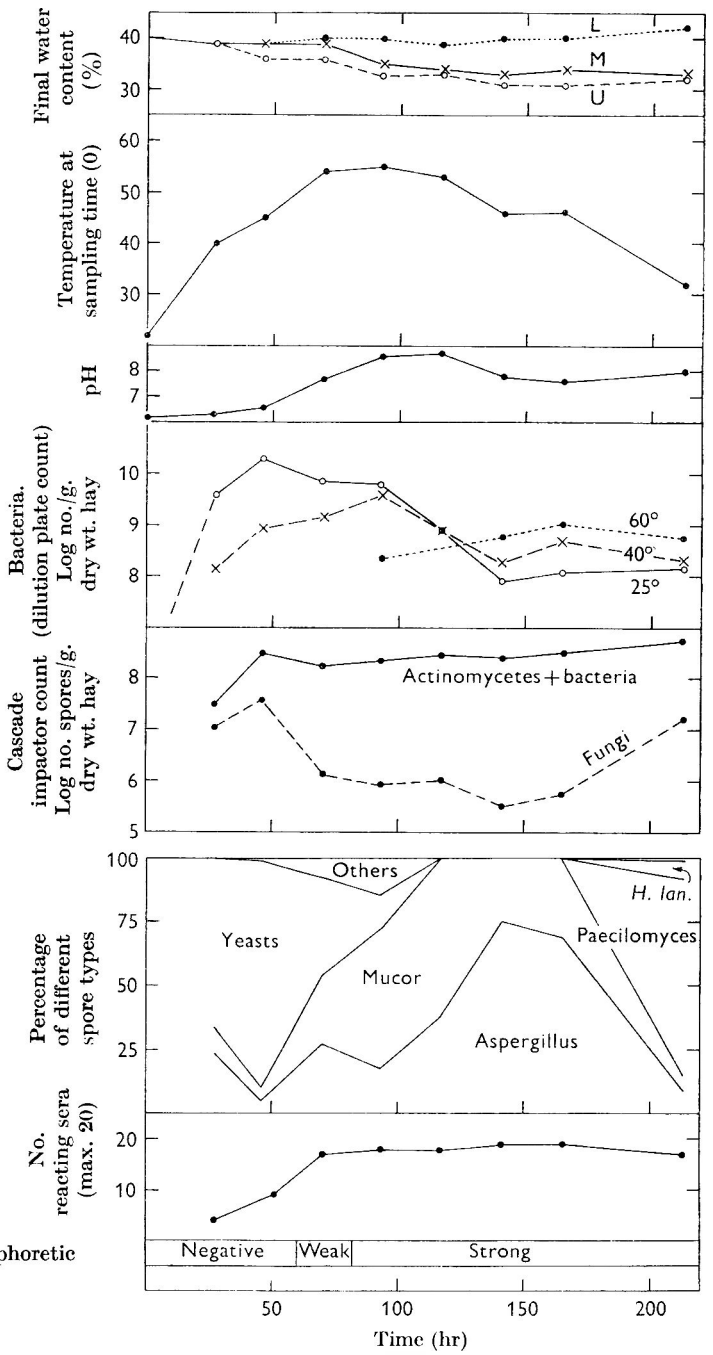

Fig. 7. Microfloral succession and development of FLH antigen in hay of 40% water content. Maximum temperatures of 62–63° were reached in 69–75 hr. L, M and U correspond to the lower, middle and upper layers of the flasks, respectively.

rare until the fourth day and reached maximum numbers on the seventh day. FLH antigen was first detectable on the third day, at the same time as the numbers of actinomycetes isolated became significant. The presence of strong immuno-electrophoretic patterns on the fourth and subsequent days relates well with the concomitant appearance of significant numbers of *T. polyspora*.

Effect of heating and growth of fungi on Thermopolyspora polyspora antigen

Gregory *et al.* (1964) found that the FLH antigen content of sequential samples of baled hay fluctuated. A possible cause of this could have been that extensive development of fungi destroyed the antigen and this possibility was studied in experiments with 50 g. quantities of 1961 hay in 2 lb. Kilner jars, as used by Pepys *et al.* (1963). Sterile ammoniated hay was inoculated with *Thermopolyspora polyspora*, incubated at 40° for 13 days and then inoculated with a mixture of *Absidia* sp., *Aspergillus fumigatus*, *Humicola lanuginosa* and *Mucor pusillus*, and incubated again for 20 days at 40°. Samples both with and without added fungi had a pH of 7·5 and extracts reacted with seventeen sera in the double-diffusion test. Typical immuno-electrophoretic patterns (A, B and C lines) were observed, though the extract of the hay inoculated with fungi was slightly less reactive than the extract of the hay inoculated with *T. polyspora* alone.

Thermopolyspora polyspora grows unevenly on hay in jars, so to ensure its equal distribution, several jars were incubated for 6 weeks at 40°, when areas of hay showing good growth were bulked, well mixed and re-distributed in four jars. Propylene oxide was added to these jars in the usual way to sterilize them and the vapour allowed to disperse (Pepys *et al.* 1963). Two of the jars were then inoculated with the mixed fungi as before, the third treated with sterile water and all three jars incubated at 40°. One jar containing the fungi was removed after 1 week and the other two after 3 weeks. The hays from all these jars as well as from the unincubated jar had similar FLH antigen contents, suggesting that prolonged incubation at 40° with or without concomitant fungal growth, did not alter FLH antigen in any way.

Self heating of barley and oat grain

In the first experiment with barley at a nominal 35% water content, the temperature slowly increased to 58·5° in 13 days, remained between 55 and 59° until 42 days, when it slowly fell to 46·5° at 55 days. There were up to 90 million Aspergillus type spores/g. dry wt. in the upper layers, mostly *A. flavus*. *Humicola lanuginosa* was most abundant in the middle layers (up to 29 million spores/g. dry wt.) but few colonies were isolated and there were few fungi in the lower layers of grain. Actinomycete spores and bacteria were most abundant in the middle and lower layers, with *Streptomyces fradiae* predominant near the surface and *Thermopolyspora polyspora* and an unidentified white actinomycete in the middle. Most *Micromonospora vulgaris* colonies were isolated towards the bottom, but this species was otherwise not abundant.

Immuno-electrophoresis showed that FLH antigen was present in extracts of barley taken from the middle layers of the Dewar flask, with pH 7·2–7·4 (Table 3). The layers above were more alkaline (pH 8·1 and 8·4) and extracts contained less

antigen; extracts of the lowest layers, with 53 and 58 % final water content, did not contain detectable amounts of antigen, but gave a non-specific reaction with all sera tested.

A second experiment with barley at 33 % water content which ended after 7 days, showed a similar slow rise of temperature as the first to 43·5°. *Aspergillus flavus* was similarly abundant in the upper layer, but the number of Penicillium colonies

Table 3. *Occurrence of FLH antigen in self-heated barley and oats grain*

Weight of sample (g.) (final wet weight)	Final water content (%)	pH	No. reacting sera (max. 20)	Immuno-electrophoretic pattern
(Expt. 1) 2·5 kg. barley (14 % water content) + 800 ml. water				
104 (top)	12	6·3	6	Negative
155	18	6·9	10	Negative
140	17	7·5	13	Negative
333	23	8·1	16	Very weak
176	27	8·4	16	Weak
212	24	7·4	17	Strong
168	26	7·2	17	Strong
291	26	7·3	18	Strong
170	38	7·3	14	Very weak
229	53	6·9	12	Negative
389 (bottom)	58	6·9	1	Negative
Control	14	6·6	—	—
(Expt. 2) 2·5 kg. barley + 700 ml. water				
242 (top)	—	—	—	—
471	30	6·4	13	C line only
1690	31	6·3	14	C line only
446	44	6·4	11	C line only
280 (bottom)	46	6·5	10	Negative
(Expt. 3) 2·5 kg. barley soaked with 1000 ml. water				
153 (top)	—	—	—	—
246	20	7·1	10	Negative
1326	33	7·1	19	Strong
1106 (bottom)	40	7·1	18	Strong
2·5 kg. oats (12 % water content) soaked with 1000 ml. water, 2·767 kg. moistened oats placed in Dewar flask				
32 (top)	—	—	—	
84	—	6·3	17	Very weak C line only
100	—	6·3	15	Very weak C line only
492	20	6·5	19	Weak C line only
293	23	6·5	19	Weak
756	28	6·6	15	Weak
239 (bottom)	38	6·6	11	Negative
Control	12	6·4	12	Negative

isolated increased with increasing depth and probably accounted for most of the 19 million/g. Aspergillus and Penicillium type spores in the lowest layer. *Absidia* sp. was also abundant in the upper layers. Aetinomycetes increased with increasing depth nearly to the bottom of the flask, but decreased again in the lowest layer. *Streptomyces fradiae* and unidentified white and grey colonies were most abundant, with a few *Micromonospora vulgaris* towards the bottom. FLH antigen was again

not detectable in extracts of the wettest lower region, with 46% final water content. Extracts of the other layers yielded only C lines which, considered in conjunction with the few *M. vulgaris* isolated, and the low pH values of the grain, may have been fungal in origin.

A third sample of barley was allowed to stand with water for 5 hr to increase its water content to 40%. It heated more rapidly than the previous samples; the temperature exceeded 60° on the eighth day and reached a maximum of 66° after 12 days. It was still over 60° when the experiment ended after 19 days. Aspergillus, Mucor and *Humicola lanuginosa* spores were all abundant in the upper layers and many *Aspergillus fumigatus* were isolated. Actinomycete spores and bacteria were most abundant in the middle layers, *Streptomyces fradiae* near the top, *Thermopolyspora polyspora* in the middle and *Micromonospora vulgaris* most abundant near the bottom of the flask. FLH antigen was not detectable in the upper layer, but strong reactions were obtained with the remainder of the grain. The lowest layer (pH 7·1) had 40% final water content and despite its large bulk (1106 g.), was uniform in appearance to the bottom of the flask. Extracts of this and the next layer above it reacted with 18 and 19 sera respectively in the double-diffusion test and both extracts gave typical patterns in the immuno-electrophoresis test.

A sample of oat grain was also allowed to absorb water to reach a water content of 37%. The temperature, which rose faster than in any of the barley samples, reached 60° after 3 days and a maximum of 64° after 10 days. It remained near 60° for a further 23 days, but then fell below 50° during the next 3 days. The pH of the grain at the end of the experiment was 6·3–6·5. *Aspergillus fumigatus* was abundant in the upper half of the flask, whereas *A. nidulans*, *Absidia* sp. and *Humicola lanuginosa* were most numerous (up to 14 million spores/g. dry wt.) in the middle layers. Actinomycetes, also most abundant in the upper half of the flask, were more abundant (up to 568 million/g. dry wt.) than in any of the barley samples. *Streptomyces fradiae* was again most abundant in the upper layers, whereas *Thermopolyspora polyspora* and *Micromonospora vulgaris* were most numerous towards the middle, with the maximum population of *M. vulgaris* slightly higher up the flask than that of the *T. polyspora*. The lowest layer with 38% final water content contained no detectable antigen and the upper layers gave reactions in the C region only in the immuno-electrophoresis tests. FLH antigen giving A, B and C lines was found in the middle of the flask. As with the second barley experiment, the scarcity of *M. vulgaris* and the low pH suggested that the C lines may have been fungal in origin.

DISCUSSION

The initial studies on the conditions favouring self-heating in Dewar flasks led to the choice of 500 g. hay in a 4 l. flask plugged with cotton-wool as the most suitable for studying the effects of varying the water content.

The importance of oxygen for the self-heating process was first shown by Miehe (1907) and also by Hildebrandt (1927); Carlyle & Norman (1941) found that a cotton plug gave satisfactory aeration of oat straw in a quart Dewar flask, with least likelihood of heat loss. The efficiency of the cotton-wool plug in allowing diffusion of air was shown by the experiment where the wooden lid was substituted

(Fig. 2). The effects of alternately starting and stopping the air flow were large, as noted by Glathe (1952) in similar experiments with chopped hay.

The maximum temperatures of 57–67° reached by 500 g. quantities of hay at 40 % water content (Fig. 3) were similar to those in field experiments with bales of 40 % water content and a stack of 30 % water content (Gregory *et al.* 1963). There was no special advantage in using 1250 g. hay in a 10 l. flask, which self-heated to similar temperatures, but required extra aeration for the hay in the lower part to heat adequately.

Table 4. *Predominant fungus and actinomycete species in hays allowed to mould at different water contents*

Water content (%)	Predominant species
26	*Aspergillus glaucus* group
28–29	*A. versicolor*, *Scopulariopsis brevicaulis*
31	*A. nidulans*, *Absidia* sp., *Streptomyces fradiae*
40	*A. fumigatus*, *Humicola lanuginosa*
47	*Humicola lanuginosa*, *Thermopolyspora glauca*, *T. polyspora*
57	*Micromonospora vulgaris*, *Paecilomyces varioti*

The final products of the Dewar flasks hays with 40 % water content were all visibly mouldy throughout the mass. The pH values of the hays were usually near 7, or above (Table 1), resembling hay from mouldy bales (Gregory *et al.* 1963) rather than brown hay from the centre of a self-heated stack, with pH values of 5 or below, which does not contain FLH antigen (Gregory *et al.* 1964). Miehe (1911) described the hay from his laboratory experiments as also quite unlike the brown hay of self-heated haystacks. The findings from our experiments, therefore, seem to apply to wet bales rather than to wet stacks, except for those particular regions in the stacks where mould had developed (Gregory *et al.* 1963).

Hildebrandt (1927) was the first to examine the microflora of self-heated hays from Dewar flasks and he isolated *Actinomyces thermophilus*, *Aspergillus fumigatus* and *Bacillus calfactor*, all previously identified by Miehe (1907) in hay. Many more species were isolated in our experiments, although *A. thermophilus* and *B. calfactor* were not recognized. Inocula of most species must be widely distributed on hay, because the same type of moulding occurred in different hays wetted to the same water content. Table 4 shows the predominant species arising at the different water contents. Thus from a knowledge of the *Aspergillus* spp. and actinomycetes present on a sample of mouldy hay, the initial water content and maximum temperature during self-heating can be assessed.

Water content is also critical for producing FLH antigen and Fig. 8 relates all the results in terms of water content. Hay of 40 % water content invariably produced FLH antigen (Table 1) and very many *Micromonospora vulgaris* and *Thermopolyspora polyspora*. Hay of 29–34 % water content gave FLH antigen in only some experiments; this range of water content is critical, because the maximum temperature reached, the numbers of thermophilic actinomycetes and the antigen content all increased greatly with small increases in water content. Hay of less than 29 % water content, which developed only C lines, showed no *T. polyspora* but there

were a few *M. vulgaris*. Hay with less than 25% water seems unlikely to develop FLH antigen. These conclusions accord with the results of field experiments of Gregory *et al.* (1964), who found that hay baled at less than 30% water content did not develop FLH antigen readily.

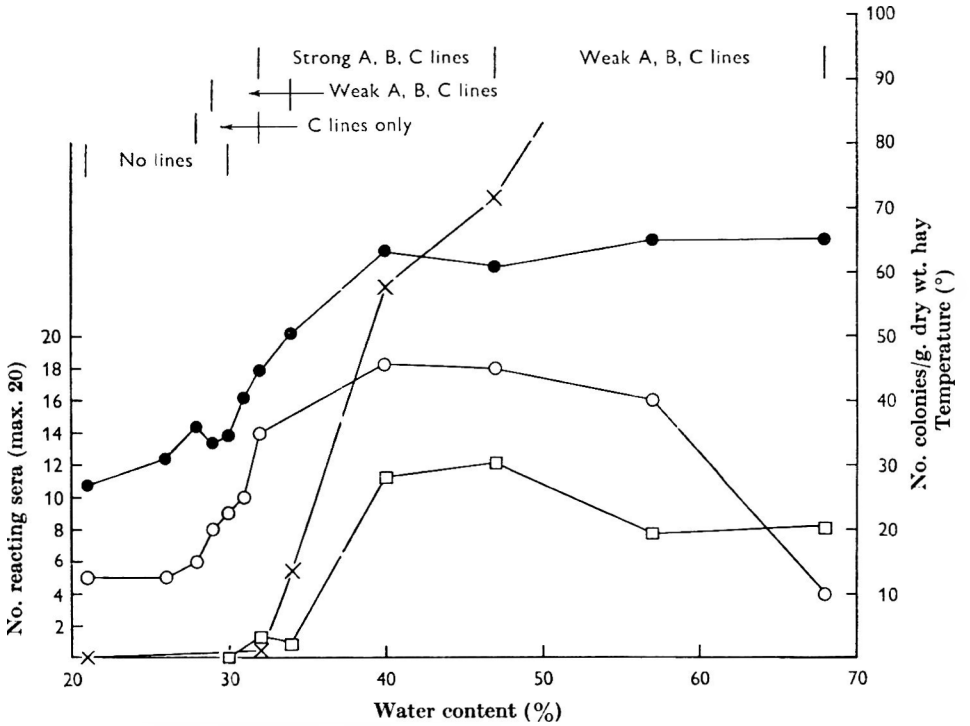

Fig. 8. Summary of results in relation to water content of hay and occurrence of FLH antigen. ●, maximum temperature; □, *T. polyspora*; ×, *M. vulgaris*; ○, No. reacting sera.

With the hays of water content of 40% and less, extracts from the lower regions of the flask reacted with more sera than those from higher up. The upper half of the flasks dried faster than the lower half (Fig. 7), so the upper layers may have become too dry to support microbial growth. In contrast, with hays of 47–68% water content, the wetter the hay, the less FLH antigen was produced (Fig. 8), particularly in the bottom of the flask (Table 2). Restricted aeration and more water in the lower layers may have adversely affected the growth of *Thermopolyspora polyspora*, which were fewer the wetter the hay (Fig. 8), but the decrease in FLH antigen seems large by comparison. *Micromonospora vulgaris* was most abundant in these wet hays.

Differences in heating pattern of hay apparently influence FLH antigen production. Table 1 shows that the old 1961 hay with multi-peaked heating pattern produced antigen more readily than 1962 hay with only one temperature maximum (Fig. 3). This may be a function of the type of hay, its age and manner of storage, or the amount of inoculum present. Fig. 3 shows that the wetted 1962′ hay took as long as 70 hr before it began to heat, but the sequential sampling study with 1962 hay (Fig. 7) showed that FLH antigen appeared after 70 hr, when the temperature

had reached 54° and was still rising. Temperature is obviously a better criterion than time after wetting of the hay to assess when antigen is likely to develop. In their field experiments done with fresh grass after cutting and partial drying, Gregory *et al.* (1964) showed that FLH antigen was detectable 4–6 days after baling, which fits in well with the results for the Dewar flask experiments done with rewetted hay.

FLH antigen can be produced in as little as 250 g. hay (Fig. 1), so that even small pockets of hay in a bale or stack, given the right conditions of water content, aeration and thermal insulation, may mould and produce antigen.

Grain can also self-heat when wet and develop a considerable microflora, including the thermophilic actinomycetes forming FLH antigen. Evidently, as with hay, FLH antigen occurs in a critical range of water content. The minimum water content for moulding of various materials including cereals, meat and textiles is generally accepted as that in equilibrium with a relative humidity of 75 % (Milner & Geddes, 1946). The equilibrium water content depends on the nature of the material and Snow, Crichton & Wright (1944*a*, *b*) estimate the safe levels for short periods of aerobic storage for wheat and oat grain and hay, as 15·7, 14·5 and 12·6 % respectively and as 14·6, 13·4 and 11·0 % respectively for long periods (at 18–20°). Christensen & Linko (1963) suggest 14·3 % as the highest water content for storing wheat safely. Waite (1949) emphasizes the importance of temperature, which affects mould growth. Hay kept at a relative humidity of 85 % (22 % water content) and a temperature of 6° (winter conditions) moulded only after 120 days, whereas at 15° (summer temperature) it moulded after 2 days; under summer conditions, 75 % relative humidity (15 % water content) and 15°, the hay did not mould. Barley and oat grain will start moulding at similar water contents to those for hay, and the safe minimum water-content for grain, to avoid producing FLH antigen, is probably the same as for hay, assessed as 25 %, but this has yet to be determined.

We thank Miss Joan Thurston for identifying grasses in the hays, and Miss Ann Macefield and Mr K. E. Fletcher for technical assistance.

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APPENDIX ON TAXONOMY

By J. LACEY

A comment is necessary on the taxonomy of some of the species isolated. To aid comparison with our earlier publications on moulding of hay and farmer's lung disease, the same names have been used throughout, although we recognized that for some species other names are preferable. Apinis (1963) and Pugh, Blakeman & Morgan-Jones (1964) propose that *Humicola lanuginosa* (Griffon & Maublanc) Bunce 1961 should be referred to *Thermomyces lanuginosus* Tsiklinsky, 1899. However, Griffon & Maublanc (1911) and Bunce (personal communication) regarded Tsiklinsky's description as insufficient to delimit the species, and Cooney & Emerson (1964) point out that the photographs, if the stated magnification is correct, show spores smaller than 3·6 μ diam. instead of the 6–10 μ of *H. lanuginosa*. Also, the photographs show only smooth spores, which are either immature, or if mature are more like *H. grisea* var *thermoides* Cooney & Emerson than the sculptured aleuriospores of *H. lanuginosa*. Tsiklinsky (1899) proposed the name *Thermomyces lanuginosus* provisionally 'en attendant que j'en aie étudié la morphologie' but no subsequent description by her has been found. Identification of isolates with *T. lanuginosus* would seem to be largely conjectural, the few known species of thermophilic fungi, and the abundance of *H. lanuginosa*, making it most likely that Tsiklinsky isolated the same species. It is questionable, however, whether such conjecture is a good basis for a taxon, and we follow Cooney & Emerson (1964) in retaining the name *Humicola lanuginosa* proposed by Bunce (1961).

A new species *Mucor miehei* was described by Cooney & Emerson (1964). This has not been distinguished from *M. pusillus* Lindt in our work, although we have now isolated it from hay. The Absidia species is probably referable to *A. corymbifera* although some isolates resembling *A. ramosa* were found and a range of intermediate forms.

Micromonospora vulgaris Waksman, Umbreit & Cordon, 1939 and *Thermoactinomyces vulgaris* Tsiklinsky 1899 are synonymous (Küster & Locci, 1964) and the latter name is generally preferred, although some of the same objections can be raised to it as to *Thermomyces lanuginosus*.

Thermopolyspora glauca Corbaz, Gregory & Lacey, 1963 is a synonym of *Thermomonospora viridis* (Schuurmans *et al.*) Küster & Locci, 1963, but the colour of the aerial mycelium could perhaps be better described as grey-blue rather than grey-green, and some of our isolates also produce black or dark brown soluble pigment. Isolates described by Corbaz *et al.* (1963) as *Thermopolyspora polyspora* have been examined by Henssen who considers it to be distinct from *T. polyspora* Henssen, 1957. It should probably be referred to *Micropolyspora* Lechevalier, Solotorovsky & McDermont 1961.

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Carotenoids and cholesterol in membranes of *Mycoplasma laidlawii*

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SUMMARY

Carotenoids synthesized by *Mycoplasma laidlawii* and cholesterol incorporated from the growth medium were confined to the cell membrane. Increased carotenogenesis induced by addition of sodium acetate to the growth medium, or inhibition of carotenogenesis by thallium acetate or diphenylamine did not affect the incorporation of cholesterol into membrane lipids. Growth of the organisms in media with various amounts of cholesterol modified the cholesterol content of the membranes with no significant effect on carotenoid formation. These results indicate that carotenoids do not interfere with the incorporation of cholesterol into *M. laidlawii* membranes.

INTRODUCTION

Mycoplasma cells are limited by a thin lipoprotein 'unit membrane' (Razin, 1963) which can be isolated following osmotic lysis of the organisms (Razin, Argaman & Avigan, 1963). The saprophytic *Mycoplasma laidlawii* strains are especially suited for preparation of membranes, because they grow faster than the other mycoplasmas, and are very sensitive to osmotic shock. Membranes of *M. laidlawii* were therefore the subject for recent studies on membrane composition (Razin, *et al.* 1963), membrane subunits (Razin, Morowitz & Terry, 1965) and enzymic activities (Pollack, Razin & Cleverdon, 1965). The results of these investigations advocate the use of Mycoplasma membranes as models for studying the properties of biological membranes in general. The ability of mycoplasmas to incorporate substantial amounts of cholesterol from the growth medium into their membrane lipids can be exploited in studies of the function of cholesterol in biological membranes. *M. laidlawii* may be especially suited for this purpose as the amount of cholesterol in its membrane can be easily regulated by varying the concentration of cholesterol in the growth medium. *M. laidlawii* was found, however, to incorporate much smaller amounts of free cholesterol than the parasitic mycoplasmas, and was practically incapable of incorporating cholesteryl esters (Argaman & Razin, 1965). *M. laidlawii* strains differ from the parasitic Mycoplasma in their ability to grow without added cholesterol (Razin & Knight, 1960) and synthesize carotenoids located in the cell membrane (Rothblat & Smith, 1961; Razin *et al.* 1963; Smith, 1963*a*). Smith (1963*b*) postulated that carotenoids in the saprophytic Mycoplasma fulfill the same roles as that of cholesterol in the parasitic strains. Among the arguments brought by Smith to support his hypothesis were the resemblance of the hydrocarbon structure

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of carotenoids and cholesterol (Smith, 1963*b*) and the finding that PPLO-serum fraction, when added to the growth medium of *M. laidlawii*, suppressed carotenogenesis (Smith, 1963*a*). This finding was interpreted by Smith as a sparing effect of cholesterol, included in the PPLO-serum fraction, on the synthesis of carotenoids. The above arguments led us to suggest that the carotenoids may interfere with cholesterol uptake by occupying the same sites in the membrane, thus explaining the low cholesterol content of *M. laidlawii* (Argaman & Razin, 1965). The purpose of the present investigation was to test this suggestion.

METHODS

Organism. *Mycoplasma laidlawii* strain B (PG 9) obtained from Dr D. G. ff. Edward (Wellcome Research Laboratories, Beckenham, Kent) was used throughout this work.

Growth conditions. The basal growth medium was tryptose broth consisting of (g/l.): Bacto-tryptose, 20; sodium chloride, 5; tris (2-amino-2-hydroxymethylpropane-1:3-diol), 5; glucose, 7; and penicillin G (crystalline) 100,000 units. The pH of the medium was 8·2 to 8·4 without adjustment. All other supplements to the growth medium were added as sterile solutions. The same batch of Difco PPLO-serum fraction was used in all experiments, to avoid variations in the cholesterol content of this medium component. Cholesterol (Calbiochem, Los Angeles, California, U.S.A.) was recrystallized twice from ethanol, dissolved in Tween 80. and added to the growth medium. Various amounts of an ethanolic solution of cholesterol (20 mg./ml.) were mixed with a constant volume of Tween 80 (10 %, v/v), heated to complete solution and added to the growth medium. By this method up to 20 mg. of cholesterol in 1 ml. ethanol could be dissolved in 1 ml. of Tween 80 (10 %, v/v), and remain in solution on addition to 1 l. of the growth medium. A solution of 10 % (w/v) bovine albumin fraction V (Calbiochem) was prepared in de-ionized water, sterilized by filtration, and added to the growth medium in a final concentration of 0·1 % (w/v) to neutralize the toxicity of the Tween 80.

Growth was done in 1 l. volumes of medium dispensed in 2 l. flasks incubated statically at 37° for 20–24 hr. The organisms were harvested by centrifugation at 9000*g* for 15 min. and washed once in *β*-buffer (Pollack, Razin, Pollack & Cleverdon, 1965) consisting of: sodium chloride, 0·15 M; tris, 0·05 M; 2-mercaptocthanol, 0·01 M in de-ionized water, adjusted to pH 7·4 with HCl.

Isolation of cell membranes. The washed organisms obtained from 1 l. medium were lysed by resuspension in 40 ml. of de-ionized water (Razin, 1964). The suspension was incubated at room temperature for 30 min. and then centrifuged at 37,000*g* for 30 min. to collect the membranes. The membranes were washed once in 40 ml. of de-ionized water and resuspended in 10 ml. de-ionized water. Membrane protein was determined in a 0·2 ml. sample of this suspension according to Lowry, Rosebrough, Farr & Randall (1951).

Estimation of carotenoids. Part of the membrane suspension (usually one-fifth) was centrifuged at 37,000*g* for 30 min. The resulting pellet was extracted with 7 ml. of boiling ethanol for 10 min. in the dark and under a nitrogen atmosphere (Rothblat, Ellis & Kritchevsky, 1954). The extracted membranes were removed by centrifugation and the extinction at 442 mμ of the supernatant fluid was measured

in a Beckman DB spectrophotometer. Extraction of the carotenoids from wet membrane material with boiling ethanol was found superior to extraction of freeze-dried membranes with chloroform + methanol (2 + 1, by vol.). The absorption spectrum of *Mycoplasma laidlawii* carotenoids measured in ethanol showed absorption maxima at 418, 442 and 472 mμ; the peak at 442 mμ was the highest. The amount of carotenoid pigments in membranes was expressed therefore as extinction at 442 mμ × 1000 per mg. membrane protein or per mg. dry-weight of membrane material.

Estimation of cholesterol. The residual part of the membrane suspension was centrifuged at 37,000*g* for 30 min. The resulting pellet was freeze-dried and weighed. Lipids were extracted from the freeze-dried material by a chloroform + methanol mixture (2 + 1, by vol.) as described previously (Argaman & Razin, 1965). Cholesterol in the lipid was separated by thin-layer chromatography and determined by the $FeCl_3$ method (Argaman & Razin, 1965).

RESULTS

Location of carotenoids and cholesterol in the cell

The confinement of the carotenoids and cholesterol to the cell membrane of *Mycoplasma laidlawii* was demonstrated in an experiment in which the same batch of organisms was divided into two equal portions. One portion was osmotically lysed and the membranes recovered quantitatively and freeze-dried. The other portion of

Table 1. *Total lipid, carotenoids and cholesterol in cells and membranes of Mycoplasma laidlawii*

Cells were grown in 2 l. of tryptose broth supplemented with 1 % (v/v) PPLO-serum fraction. The cells were harvested after 24 hr incubation at 37°, washed twice in β-buffer and divided into two equal portions. One portion was osmotically lysed and the membranes recovered quantitatively and freeze-dried. The other portion of the cells was freeze-dried without any prior treatment. Total lipid, carotenoids and cholesterol were determined in the dried material as described under Methods.

	Total dry wt. (mg.)	Total lipid (mg.)	Carotenoids (A_{442} × 1000/mg. lipid)	Cholesterol (μg./mg. lipid)
Whole cells	221·9	25·8	65·1	22·3
Membranes	73·0	26·0	64·6	23·4

cells was freeze-dried without previous treatment. Table 1 shows that the membrane fraction, which constituted about 32 % of the total dry weight of the organisms, contained practically the same amount of total lipid, carotenoids and cholesterol as the whole organisms, Hence, carotenoids and cholesterol seem to be exclusive constituents of the cell membrane of *M. laidlawii*.

Effect of variations in carotenogenesis on cholesterol uptake

Rothblat & Smith (1961) reported that the addition of 0·5 % (w/v) of sodium acetate to the growth medium increased carotenoid formation by *M. laidlawii*. Figure 1 shows that the addition of increasing amounts of sodium acetate to tryptose broth increased pigment formation very markedly. The increase in the caro-

tenoid content of the membranes had no significant effect on the amount of cholesterol incorporated into the membranes from the growth medium. Sodium acetate showed some growth inhibition, reflected by the decrease in the yield of membrane material (Fig. 1).

Table 2. *Effects of inhibitors of carotenogenesis on the cholesterol content of Mycoplasma laidlawii membranes*

The organisms were grown in 1 l. volumes of tryptose broth supplemented with 1 % (v/v) PPLO-serum fraction and the inhibitor tested.

Inhibitor in growth medium	Yield of membranes (mg. dry wt.)	Carotenoids ($E_{442} \times 1000$/mg. dry wt. of membranes)	Cholesterol (μg./mg. dry wt. of membranes)
Thallium acetate			
0·025 % (w/v)	10·1	2·1	7·4
0·050 % (w/v)	7·1	1·6	7·7
Diphenylamine			
6×10^{-5} M	10·6	13·6	8·0
$1·2 \times 10^{-4}$ M	8·4	7·1	8·5
No inhibitor	17·8	24·4	8·5

Thallium acetate was found to inhibit markedly pigment formation by *Mycoplasma laidlawii* (observed originally by J. D. Pollack). Diphenylamine also decreased significantly the amount of carotenoid pigments produced by the organisms. Both agents inhibited the growth of the test organism as well (Table 2). Inhibition of pigmentation by diphenylamine was reversible to a certain extent, since the organisms which were completely devoid of pigment when harvested, turned yellow on subsequent washings. Decreased of pigmentation by the inhibitors was not accompanied by any significant change in the amount of cholesterol incorporated into the cell membrane (Table 2).

Effect of variations in the cholesterol content of the membranes on carotenogenesis

PPLO-serum fraction serves as a source for cholesterol in many growth media for Mycoplasma. The amount of cholesterol incorporated into the membranes of *Mycoplasma laidlawii* increased from nearly zero in the tryptose medium without PPLO-serum fraction, to a maximum of about 8 μg./mg. dry weight of membranes when the concentration of PPLO-serum fraction in the medium reached 2 % (v/v). Some decrease in the carotenoid content of the membranes was noticed when the concentration of PPLO-serum fraction in the medium was increased, confirming previous results of Smith (1963*a*). PPLO-serum fraction improved the growth of *M. laidlawii* in tryptose broth, as reflected by the increase in membrane yield (Fig. 2).

To test whether the decrease in carotenoids caused by PPLO-serum fraction was dependent on its cholesterol component, cholesterol itself was added to the growth medium instead of PPLO-serum fraction. Table 3 shows that the amount of cholesterol incorporated into the membranes increased with the increase of its concentra-

tion in the growth medium, reaching about the same value (above 7 μg cholesterol/mg. dry weight of membranes) obtained with PPLO-serum fraction. The amount of carotenoids in the membranes was not affected to any significant degree by the changes in the cholesterol content of the membranes (Table 3).

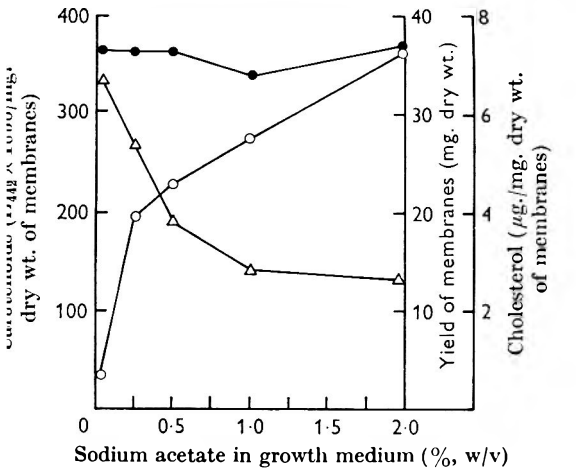

Fig. 1

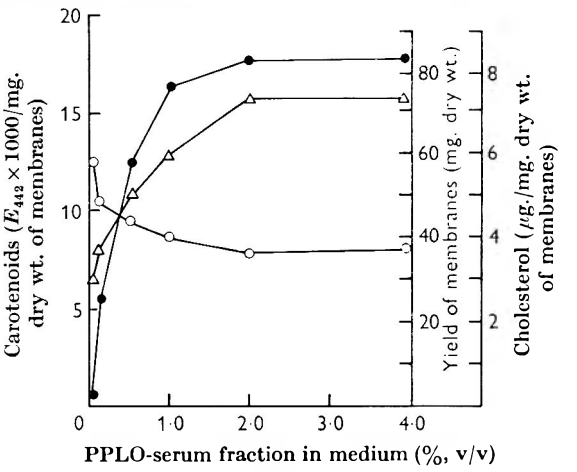

Fig. 2

Fig. 1. Effect of sodium acetate added to the growth medium on the carotenoid and cholesterol content of *Mycoplasma laidlawii* membranes. The organisms were grown in 1 l. volumes of tryptose broth supplemented with 1% (v/v) PPLO-serum fraction and various amounts of sodium acetate. Cholesterol (●); carotenoids (○); membrane yield (Δ).

Fig. 2. Effect of PPLO-serum fraction added to the growth medium on the carotenoid and cholesterol content of *Mycoplasma laidlawii* membranes. The organisms were grown in 1 l. volumes of tryptose broth supplemented with various amounts of PPLO-serum fraction. Cholesterol (●); carotenoids (○); membrane yield (Δ).

Table 3. *Effect of cholesterol added to the growth medium on the carotenoid and cholesterol content of Mycoplasma laidlawii membranes*

The organisms were grown in 1l. volumes of tryptose broth supplemented with 0·1% (w/v) bovine albumin fraction V and various amounts of cholesterol dissolved in Tween 80. The final concentration of Tween 80 in the medium was 0·01% (v/v).

Cholesterol added to medium (μg./ml.)	Yield of membranes (mg. dry wt.)	Carotenoids (E_{442} × 1000/mg. dry wt. of membranes)	Cholesterol (μg./mg. dry wt. of membranes)
0	57·2	23·1	0·3
0·5	66·0	26·1	2·8
2·5	64·8	20·8	4·7
5·0	52·8	24·6	7·2
10·0	48·3	23·8	7·8
20·0	42·7	20·2	7·1

DISCUSSION

Cholesterol and carotenoids seem to be restricted to the cell membrane in *Mycoplasma laidlawii*. Rothblat & Smith (1961) reported that only about 70% of the cell cholesterol and 80% of the carotenoids could be found in the insoluble fraction

or 'membrane fraction' of *M. laidlawii* and the rest in the 'soluble fraction'. In light of the recent work of Pollack *et al.* (1965) it seems that the 'soluble fraction' analysed by Rothblat & Smith contained small membrane fragments, not sedimentable by centrifugation at 30,000*g* for 20 min., resulting from disruption of the delicate membranes by the sonic treatment. Osmotic lysis of the organisms, used in the present work, has been shown to be preferable for the separation of membranes from other cell constituents (Pollack *et al.* 1965). Our data show that *M. laidlawii* under the growth conditions tested was capable of incorporating cholesterol up to a value of 7–8 μg/mg. dry weight of membranes, corresponding to about 3 % by weight of the total membrane lipids. Thus, cholesterol seems to be a minor component of *M. laidlawii* lipids. In parasitic Mycoplasma cholesterol was found to constitute up to one third of membrane lipids (Argaman & Razin, 1965). The carotenoid pigments are apparently also minor components of *M. laidlawii* lipids. Preliminary experiments to determine the pigments gravimetrically, following chromatographic separation, have indicated that they constitute 1–3 % of the total membrane lipids (Razin, unpublished results).

No attempt was made in the present work to determine non-pigmented, more saturated, carotenoids in *Mycoplasma laidlawii*. They were certainly present in organisms grown with diphenylamine, known to inhibit the final oxidative stages in carotenogenesis leading to formation of the more unsaturated pigmented products (Cho, Corpe & Salton, 1964; Rilling, 1965). Removal of the inhibitor during the washing of organisms and derived membranes resulted in some pigment formation, apparently due to oxidation of the saturated carotenoids accumulated during growth with diphenyl-amine. We do not as yet know the stage or stages of carotenogenisis inhibited by thallium acetate. The marked reduction in growth of *M. laidlawii* caused by thallium acetate, in concentrations used routinely in selective media for Mycoplasma, suggests the omission of this agent from growth media for Mycoplasma whenever possible.

Our experiments did not show any relationship between the amount of carotenoid pigments and that of cholesterol in *Mycoplasma laidlawii* membranes. The amount of either of these components could be markedly changed without affecting that of the other. Decreased carotenogenesis following the increase in PPLO-serum fraction concentration in the growth medium does not involve the cholesterol component of this medium supplement. The lower capacity of the saprophytic Mycoplasma to incorporate cholesterol does not seem thus to depend on the presence of carotenoid pigments in their membranes. The explanation for this phenomenon may rather involve differences in phospholipid composition between the saprophytic and parasitic strains. Phospholipids form the major lipid component of Mycoplasma membranes (Morowitz *et al.* 1962; Razin *et al.* 1963). The two major fractions of *M. laidlawii* strain B phospholipids were found to contain covalently bound glucose (Smith & Henrikson, 1965) differing from the phospholipid composition of the parasitic *M. gallisepticum* as reported by Tourtellotte, Jensen, Gander & Morowitz (1963).

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Recent Research in Molecular Biology. Edited by R. C. CLOWES. Published by British Medical Bulletin, Vol. 21, No. 3, 95 pp. Price 30*s.*

Heinemann Pharmaceutical Monograph, Vol. 3. *Sterilization and Disinfection.* By T. D. WHITTET, W. B. HUGO and G. R. WILKINSON. Published by William Heinemann Medical Books Ltd., 23 Bedford Square, London, W.C. 1. Price 30*s.*

Chemical Microbiology. By A. H. ROSE. Published by Butterworth and Co. Ltd., 88 Kingsway, London, W.C. 2. 247 pp. Price 37*s.* 6*d.*

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THE SOCIETY FOR GENERAL MICROBIOLOGY

The Society for General Microbiology held its forty-fourth General Meeting at King's College, Aberdeen, on Thursday, Friday and Saturday 16, 17 *and* 18 *September* 1965. *The following communications were made:*

ORIGINAL PAPERS

The Metabolism of Aromatic Compounds by Yeasts and Moulds. By R. F. Bilton and R. B. Cain (*Microbiology Group, Department of Botany, The University of Newcastle upon Tyne*)

Apart from the studies with *Neurospora crassa* (Gross, S. R., Gafford, R. D. & Taturn, E. L. (1956), *J. biol. Chem.* **219**, 781), the way in which fungi utilize aromatic compounds has received little attention.

Several yeasts and yeast-like moulds have been examined for enzymic patterns of aromatic ring degradation.

Ability to grow upon an aromatic substrate seems widely distributed among the yeasts, but two groups are apparent: (*a*) strains of *Saccharomyces cerevisiae*, *S. pastori* and *Candida tropicalis* utilize only catechol and resorcinol of some 30 compounds tested; while (*b*), a larger group, *Rhodotorula mucilaginosa*, *Pichia membranaefaciens*, *Sporobolomyces* spp., *Debaryomyces subglobosus*, *D. hanseni*, a torula-like organism VC4, two unidentified yeasts IC and YX, and a *Fusarium* spp. utilize these aromatic compounds and also show growth on some or all of *p*-hydroxybenzoate, protocatechuate, vanillate, quinate, ferulate, *p*-coumarate and chlorogenate. Washed cells of group (*b*) grown on *p*-hydroxybenzoate, oxidized *p*-hydroxybenzoate and protocatechuate without any lag; catechol is similarly oxidized by the *Debaryomyces* spp. and yeast IC but the remainder of the group show an adaptive response for catechol oxidation. Cell-free extracts of all isolates, when suitably induced, oxidize protocatechuate to β-ketoadipate, which was characterized chemically from oxidations by *Sporobolomyces*, *Rhodotorula* and *Fusarium* and detected chromatographically in the products of every other organism.

None of the yeast isolates cleave protocatechuate by a 4,5-oxygenase type of splitting (Dagley, S. *et al.* (1964), *Nature, Lond.* **202**, 775), although we have isolated a *Penicillium* spp. which forms pyruvate, a recognized end-product of this type of ring fission from protocatechuate.

Spectrophotometric studies have shown that the yeasts and *Fusarium* which degrade protocatechuate do so through β-carboxymuconic acid and β-earboxymuconolactone and thus resemble *Neurospora crassa*. None of 70 strains of bacteria examined have this lactone as an intermediate.

The *Debaryomyces* spp. and yeast IC are anomalous in that the extracts of *p*-hydroxybenzoate-grown cells oxidize catechol (but not protocatechuate), through *cis-cis*-muconate and (+)-muconolactone to β-ketoadipate. There seems, on the present evidence, to be a clear difference in the routes by which fungi and bacteria form β-ketoadipate from protocatechuate.

Utilization of aromatic Sulphonic Acids by Micro-organisms. By D. R. Farr and R. B. Cain (*Microbiology Group, Department of Botany, The University of Newcastle upon Tyne*)

About a dozen strains of *Pseudomonas fluorescens*, isolated from sewage, were capable of growing upon benzenesulphinic, benzenesulphonic and *p*-toluenesulphonic acids as sole source of carbon. The molar growth yields obtained on these substrates, however, were low

when compared with the yields when grown on benzoic acid. None of the isolates grew upon ethylbenzenesulphonate or the corresponding (detergent) derivatives with 4-18 C atoms in the side-chain. Growth of the organisms was correlated with disappearance of ring structure and appearance of the sulphonate group as sulphite ion (Simpson, J. R. (1954), M.Sc. Thesis, University of Wales). Washed cells of two strains, A and B, oxidized only 46% of benzenesulphonate and toluenesulphonate; the remainder was presumably assimilated because no aromatic substrate was detectable at the cessation of oxygen uptake.

Cell extracts from isolate A oxidized catechol with the transient formation of 2-hydroxymuconic semialdehyde, which was isolated and characterized chemically. On further incubation the same extracts degraded this intermediate to acetaldehyde and pyruvate. In contrast extracts of strain B degraded catechol via *cis-cis*-muconate and (+)-muconolactone to β-ketoadipate. Five of our original 12 isolates resembled strain A, the remainder strain B, although all strains were obtained from the same sewage sample.

The nature of the growth substrate markedly affected the route of ring fission adopted by strain A. Growth upon fumarate yielded extracts with no catechol oxygenase activity of either type; benzoate induced 1,2-oxygenase cleaving catechol to *cis-cis*-muconate whilst growth upon benzenesulphonate or *p*-toluenesulphonate induced 2,3-oxygenase yielding 2-hydroxymuconic semialdehyde from catechol.

Growth upon *p*-toluenesulphonate induced in washed cells of strain A the ability to oxidize only catechol and 3- and 4-methylcatechols. Possible intermediates such as *p*-cresol, *p*-hydroxybenzoate and protocatechuate were oxidized only after long periods. Extracts prepared from these cells oxidized 3- and 4-methylcatechols by a route analogous to the oxidation of catechol, yielding the corresponding methyl-2-hydroxymuconic derivatives with characteristic absorption spectra in acid and alkali.

Utilization of Halogenated Aromatic Compounds by *Nocardia erythropolis*. By A. Smith and R. B. Cain (*Microbiology Group, Department of Botany, The University of Newcastle upon Tyne*)

Halogen analogues of benzoic and *p*-nitrobenzoic acids were incapable of supporting growth of *Nocardia erythropolis* which had been induced to utilize benzoate or *p*-nitrobenzoate respectively. Growth of the organism on benzoate and *p*-nitrobenzoate was suppressed by the presence of halogen analogues, the fluoro-analogue being a more potent inhibitor than the chloro-derivative.

Benzoate and *p*-nitrobenzoate-grown cells readily oxidized the halogen-analogues of the original substrates in order of effectiveness: fluoro- > chloro- > bromo- > iodo-. Conversely, halogen analogues inhibited oxidation of the normal substrates, the fluoro-compounds being the most effective inhibitors and the bromo-analogues having negligible effect.

The respective halogen-analogues acted as non-metabolizable enzyme inducers for benzoate and *p*-nitrobenzoate oxidation and were also observed to accelerate enzymic induction of the organism to the authentic substrate in the case of benzoic acid; however, they retarded enzyme induction in the case of *p*-nitrobenzoic acid.

The inhibitory effects of fluoroacetate and fluorocitrate on aromatic substrate oxidation suggest that the fluoro-aromatics are degraded to fluoroacetate. Chromatographic evidence for the production of fluoroacetate and traces of fluorocitrate from 2-fluoro-4-nitrobenzoate by dried cell preparations of *N. erythropolis* has been obtained.

An Extracellular Lipopolysaccharide from a Lysine-requiring Mutant of *Escherichia coli*. By K. W. Knox, Alina Taylor and Elizabeth Work (*Twyford Laboratories, Twyford Abbey Road, London, N.W. 10*)

Lysine-requiring mutants of *Escherichia coli* which lack diaminopimelic acid decarboxylase are known to accumulate diaminopimelic acid and other compounds, including lipid, when grown under lysine-limiting conditions; they also show biphasic growth (Lilly, M. D., Clarke, P. H. & Meadow, P. M. (1963), *J. gen. Microbiol.* **32**, 103; Municio, A. M.,

Diaz, T. & Martinez, A. (1963), *Biochem. Biophys. Res. Commun.* **11**, 195). The lipid produced under such conditions was shown to be an antigenic lipoglycopeptide by Bishop, D. G. & Work, E. (1965), *Biochem. J.* **96**, 567). Heating the lipoglycopeptide in 45% aqueous phenol at 68° for 15 min. yielded a lipopolysaccharide which was purified by precipitation with magnesium ions.

The carbohydrate components of the lipopolysaccharide have been identified and estimated; they are L-glycero-D-mannoheptose, D-glucose, D-galactose, glucosamine and 2-keto-3-deoxy-octonic acid. In addition, the preparation contains ethanolamine, phosphate, lauric acid, myristic acid, β-hydroxy-myristic acid and small amounts of palmitic acid. These components are typical of lipopolysaccharides isolated from the endotoxins of Gram-negative bacteria; further, the material has the biological properties of endotoxin, being pyrogenic, toxic and an effective adjuvant. Mild acid hydrolysis of lipopolysaccharide yielded a phosphorylated polysaccharide containing heptose, glucose and galactose, and an insoluble fraction with the composition typical of lipid A from endotoxin. These results will be compared with those reported for lipopolysaccharides from cells of rough strains of *E. coli* and *Salmonella*.

The rate of appearance of lipoglycopeptide in the culture fluid during the growth cycle has been determined by the quantitative precipitin test, using homologous antiserum. Lipoglycopeptide is not detectable until after growth has ceased owing to lysine depletion (6 hr.). During the subsequent period of 16 hr, when there is no increase in cell mass, lipoglycopeptide is released at a linear rate; the resumption of growth did not change the rate of production of lipoglycopeptide. Cells collected at intervals during growth have been examined under the electron microscope and the results are reported by Vesk, M., Knox, K. W. & Work, E. (1965), *Proc. Soc. gen. Microbiol.* following abstract).

The Effect of Lysine Limitation on the Morphology of a Lysine-requiring Mutant of *Escherichia coli*. By Maret Vesk, K. W. Knox and Elizabeth Work (*Twyford Laboratories, Twyford Abbey Road, London, N.W.* 10)

Escherichia coli ATCC 12408, a lysine-requiring mutant, was grown under conditions of lysine limitation and the rate of appearance of extracellular lipoglycopeptide followed (Knox, K. W., Taylor, A. & Work, E. (1965), *Proc. Soc. gen. Microbiol.*, abstract above). As a control, cells were grown in the presence of excess lysine, when extracellular lipoglycopeptide was not formed.

Samples of control and lysine-limited cells were taken at regular intervals up to 26 hr and examined under the electron microscope, using the techniques of negative staining and thin sectioning. To observe any material adhering to the cells, cells were not washed prior to fixation. Lysis was not observed in the control or lysine-limited cells. Cytoplasmic membranes were present in both control and lysine-limited cells. Samples of lysine-limited cells contained a number of extracellular globules in close association with the cells, the amount increasing with the age of the cells. These globules were identical with those deposited from culture medium by centrifugation at 100,000*g* for one hour. In thin sections, the globules were seen to be formed wholly or partially from some of the cell-wall components.

The morphology of the lysine-limited cells is discussed, and the multilayered structure of the cell wall of both lysine-limited and control cells is compared with structures observed by other authors.

Growth Phase and Mating Ability Relationships in *Escherichia coli* K12. By K. W. Fisher (*Department of Physics, Kansas State University, Manhattan, Kansas* 66504, *U.S.A.*; present address *Medical Research Council, Microbial Genetics Research Unit, Hammersmith Hospital, London, W.* 12)

Mating experiments in *Escherichia coli* K12 have usually employed logarithmic phase cells. In the present experiments cultures of Hfr cells have been examined at various times after re-inoculation from a stationary phase culture into fresh warm growth medium.

Their ability to form zygotes as well as their ability to adsorb and support growth of the male specific phage $\mu 2$ (Dettori, Maccaearo & Piccinin (1961), *Giornale di Microbiol.* **9**, 289) has been determined. The results obtained indicate that lag phase cells, immediately after inoculation, show a low probability of zygote formation and adsorb phage $\mu 2$ with a low efficiency. At approximately the time at which cell division is initiated (determined by counting with a Coulter counter) the ability of male cells (Hfr) to perform both functions increases until the culture has entered the stationary phase. With increasing time in the stationary phase there is a decrease in both the aspects of maleness measured in these experiments. Furthermore, the period of maleness can be extended by diluting a logarithmically growing male culture with fresh warm medium. These results lend support to the idea that the male cell performs a process or possesses a structure essential to mating and $\mu 2$ plating, such as the sex fimbriae described by Brinton, Gemski & Carnahan ((1964), *Proc. nat. Acad. Sci, Wash.*, **52**, 776). Consistent with this hypothesis are observations that male cells can be temporarily emasculated either by violent agitation before mating or by separation of mating pairs. The latter treatment prohibits mating with a second female F^- cell. Either type of damage can be repaired by a short period of growth.

Experiments with recipient F^- cells indicate there is a higher efficiency of zygote formation when logarithmic phase F^- cells are employed.

A Survey of the Distribution of Poly-β-hydroxybutyrate in *Azobacter* and Related Genera. By H. Stockdale, D. W. Ribbons* and E. A. Dawes (*Department of Biochemistry, University of Hull.*

The presence of poly-β-hydroxybutyrate (PHB) has been reported previously in *Azotobacter chroococcum*, (Lemoigne, M. & Girard, H. (1943), *C.R. Acad. Sci., Paris*, **217**, 557), *A. vinelandii* (Forsyth, W. G. G., Hayward, A. C. & Roberts, J. B. (1958), *Nature, Lond.* **182**, 800) and *A. agilis* (Sobek, J. M. & Clifton, C. E. (1962), *Proc. Soc. exp. Biol. Med.* **109**, 408).

An investigation was carried out with representative strains of all known species of the genera *Azotobacter*, *Beijerinckia*, *Derxia* and *Azotomonas*, to determine the distribution of PHB throughout this group, and to see if possession of it could be used as a criterion for classification. All strains of *Azotobacter*, *Beijerinckia* and *Derxia* grew well at 30° in Norris's nitrogen-free medium with 1% glucose and laid down PHB, ranging from 5 to 70% of the dry weight. The authenticity of the PHB samples isolated was checked by elemental analysis, and infra-red spectra of polymer films. PHB was assayed by the method of Law, J. H. & Slepecky, R. A. ((1961), *J. Bact.* **82**, 33). Interest in the remarkably high PHB content of *A. beijerinckii* led us to carry out further work on this organism.

The only available strain of *Azotomonas*, namely *A. insolita* NCIB 8627, showed no growth on nitrogen-free medium. Growth was luxuriant on nutrient broth with 1% glucose, at 30°. No PHB could be detected in the lipid fraction of this organism, either immediately after harvesting or after incubation for 24 hr in nitrogen-free medium with 3% glucose.

During growth, the polymer content of the organisms followed a fairly standard pattern, remaining reasonably constant during exponential growth and reaching a peak in the early stationary phase, after which it declined, rapidly at first, and later more slowly.

An investigation of the infra-red spectra of organisms depleted of their PHB reserves by starvation was also carried out. Samples were freeze dried and pressed into KBr discs. No significant spectral differences were found amongst the members of the group.

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A Trial of Association Methods for Selecting Determinative Characters from a Collection of Micrococcaceae Isolates. By E. B. Pike (*Bacteriology Department, West of Scotland Agricultural College, Auchincruive, Ayr*)

The choice of taxonomic boundaries and key characters for the aerobic Micrococcaceae is controversial (Cowan, S. T. (1962), *J. appl. Bact.* **25**, 324). Statistical association methods have been used to select key characters for bacteria (Levine, M. (1918), *J. Bact.* **3**, 253) and the author decided to apply similar treatment to a study of 41 characters displayed by 73 strains of Micrococcaceae freshly isolated from dust, skin and cheese. Data handling was performed manually with punched cards. Association was measured between all valid combinations of character pairs using Yule's coefficient, Q; individual Q values were fused into a 'characters × characters' matrix. A strip method of cluster analysis (Sneath, P. H. A. (1957), *J. gen. Microbiol.* **17**, 201) was used to obtain a rearranged order of characters in the matrix, so that characters with similar distributions of Q values were placed adjacently. The re-arranged matrix displayed four square clusters (A, B, C and D) of positively associated character pairs arranged along the principal diagonal; these subtended clusters of negatively associated pairs vertically and horizontally from their sides. The characters in these clusters were assumed to define the specific positive and negative character reactions of four warrantable taxa latent in the collection of strains, while the cluster distribution were assumed to reflect taxonomic relations.

Cluster A was large and distinct; its positively associated characters have been attributed to *Staphylococcus epidermidis*. Central characters were growth in 15% (w/v) NaCl broth, glucose fermented anaerobically, and acid production from glycerol. Clusters B, C and D showed overlapping of positively associated characters which appeared to define: cluster B, *Micrococcus roseus* (central characters: pink pigment, acid from inulin, dextrins hydrolysed); cluster C, *M. luteus* or *Sarcina lutea* (starch hydrolysed, lemon pigment, tetrads) and cluster D, *S. afermentans* Shaw, Stitt & Cowan (no attack on glucose, heaped rough colony, packets).

Variation in Spore Morphology in a Streptomycete. By W. Hodgkiss and T. G. Mitchell (*Torry Research Station, Aberdeen, Ministry of Technology*)

Electron microscopy of the surface structure of streptomycete spores has been reported by Flaig *et al.* ((1952), *Plant & Soil*, **4**, 118); Baldacci & Grein ((1955), *G. Microbiol.* **1**, 28); and Ettlinger *et al.* ((1958), *Arch. Mikrobiol.* **31**, 326). The spores of 600 strains examined by Tresner *et al.* ((1961), *J. Bact.* **81**, 70) were found to conform to four types—smooth, warty, spiny and hairy. These authors considered that spore surface structure was a constant feature of considerable taxonomic value.

During participation in the International Streptomyces Project (I.S.P.) it was noticed that spore preparations of *Streptomyces griseomycini* (I.S.P. 5159), grown on yeast extract-malt extract agar (I.S.P. medium 2) showed smooth and spiny spores on the same sporophore. Further examinations were made at weekly intervals of cultures grown at 25° on this I.S.P. medium 2, on oatmeal agar (I.S.P. medium 3), on starch salts agar (I.S.P. medium 4) and on glycerol-asparagine agar (I.S.P. medium 5). The nature of the medium considerably influenced the morphology of spores. The ratio of spiny: smooth spores varied from 92:8 on medium 4 at 3 weeks to 1:99 on medium 2 at 1 week. Development of aerial mycelium was extremely slow on medium 5; spores were first detected after 4 weeks incubation and all these spores were smooth. On media 2, 3 and 4 the peak spiny: smooth spore ratio was reached at 3 weeks; the relative number of spiny spores declined thereafter. The spiny appearance was most consistent on medium 4; on media 2 and 3 spines showed variation in size and in numbers per spore.

The occurrence of very occasional smooth spores amongst the usual spiny spores of *S. viridochromogenes* was noted by Lechevalier & Tikhonenko ((1960), *Mikrobiologiya*, **29**, 43). Preobrazhenskaya *et al.* ((1960), *Mikrobiologiya*, **29**, 51) reported that some strains of *S. coerulescens* possessed smooth spores although these strains did not differ in cultural characteristics from spiny-spored strains.

Results indicate that some of the reasons for variation in spore morphology in *S. griseomycini* are the type of medium employed, the rate of development of spines and possibly the relative fragility of these spines.

Inter-oceanic Relationships of Tropical Phytoplankton. By E. J. Ferguson Wood (*Institute of Marine Science, University of Miami, Florida*)

In a study of tropical phytoplankton from the Indian, Pacific and Atlantic Oceans, one is struck by the similarities that occur, both in regard to the distribution of numbers and of species. The greatest preponderance of diatoms occurs in regions close to shore, especially in areas of heavy rainfall such as the Timor, Java and Arafura Seas, the northern Coral Sea, the coast of Venezuela and the Gulf of Guinea and in regions of upwelling such as the north-eastern Indian Ocean and the Benguela current south of Sao Thome. In the open oceans, dinoflagellates especially naked dinoflagellates or coccolithophores, are much more numerous than diatoms. The relative numbers of coccolithophores and naked dinoflagellates varies in different regions but so far no correlations with hydrological conditions have been made. Coccolithophores predominate in the Gulf of Guinea, the Timor Sea and the Straits of Florida, dinoflagellates in the north-eastern Indian Ocean between Sumatra and Western Australia, in the Amazon region and the north coast of Brazil and in the Coral and northern Tasman Seas. 'Red tide' blooms of the blue-green *Trichodesmium erythraeum* occur repeatedly in certain areas such as the Indian Ocean north-west of Freemantle, the Java and Timor Seas, the Coral Sea, Gulf of Mexico and western tropical Atlantic. Causes of these intense blooms are as yet unknown.

Organic Aggregation in Sea water by Alkaline Precipitation of Inorganic Nuclei during the Formation of Ammonia by Bacteria. By J. McN. Sieburth (*Narragansett Marine Laboratory, University of Rhode Island, Kingston, U.S.A.*)

The dominant form of particulate organic matter in the ocean is in the form of non-living 'organic aggregates' (Riley, G. A. *et al.* (1964), *Limnol. & Oceanogr.* **9**, 546) which presumably are derived from dissolved organic matter by bubble extraction in surface waters (Sutcliffe, W. H., Jr. *et al.* (1963), *Deep-Sea Res.* **10**, 233). However, the widespread occurrence of aggregates throughout the water column with apparent concentration in bottom water indicates that other mechanisms may occur.

While using 'magnesium hydroxide' coprecipitation (Jeffrey, L. M. & Hood, D. W. (1958), *J. Mar. Res.* **17**, 247) to concentrate trace quantities of aromatic compounds from sea water, the efficacy for organic aggregation became apparent. Only slight alkalinization is required since precipitates form at pH's approaching 9. Such alkalinization must occur (at least microzonally) when ammonia is liberated during protein decomposition by bacteria. Experimental observations indicated that this mechanism of organic aggregation can occur.

Without bubbling, natural bacterial populations formed aggregates in sea water when either soluble or insoluble proteinaceous substrates were decomposed to produce ammonia. Naturally, chemically, and bacterially produced aggregates were indistinguishable microscopically, stained similarly with Gram stain and dissolved upon acidification below pH 3. Emission spectroscopy indicated that Ca was dominant in naturally and bacterially formed aggregates while Mg was higher in chemically produced aggregates. Fe and Al were also present in all preparations. Complex mineral assemblages have recently been shown to be associated with organic aggregates (Chave, K. E. (1965), *Science*, **148**, 1723). If the mechanism described is operative in the sea then bacteria would play a central role in the food web by taking organic matter out of solution and providing aggregates for filter feeders and for further bacterial activity which would release inorganic nutrients for phytoplankton.

Other processes may also be involved in the formation of naturally and bacterially produced aggregates.

Micro-organisms and Organic Matter Attached to the Surfaces of Marine Sand Grains. By J. G. Anderson and P. S. Meadows (*Department of Zoology, University of Glasgow*)

Little detailed information is available on the distribution and abundance of organic matter and micro-organisms on the surface of marine sand grains. We have therefore studied sand from intertidal sand flats, to obtain some estimate of the organic matter and micro-organisms present. The sand used was clean to the naked eye, and had an average particle diameter ranging from 0·1 to 0·5 mm. Our experiments fell into three groups.

(*a*) Treatment with de-ionized or distilled water causes many bacteria to detach from the surface of marine sand grains (Meadows, P. S. (1964), *J. exp. Biol.* **41**, 499–511), and so provides a useful method of estimating the abundance of bacteria on the surfaces of marine sand grains. Sands were therefore shaken vigorously in de-ionized water for 3–5 min. and viable counts, total counts, and optical density measurements were made on the resultant washings. Viable counts ranged from 4 to 40 bacteria/mm.^{2} of sand grain surface, and total counts from 2×10^4 to 1×10^5/mm.^{2}. Total counts were hence about $\times 1000$ as great as viable counts. This difference was not due to mortality induced by the de-ionized water.

(*b*) Samples of washings obtained as in (*a*) were evaporated almost to dryness on microscope slides, fixed in OsO_4 vapour and stained. Microscopic examination of the stained washings showed that, besides many bacteria, there were slabs of faintly staining material, and many diatoms.

(*c*) Sand grains were fixed in OsO_4 vapour, stained, and examined microscopically. On their surfaces were many bacteria and algae, and stalked organisms of the same size as stalked protozoa. Some parts of the surfaces were covered by intensely staining organic material, while others were bare and transparent.

Growth Dynamics of a Marine Photosynthetic Flagellate under Continuous Flow Conditions in Limiting Vitamin B_{12} Concentration. By M. R. Droop (*Marine Station, Millport, Scotland*)

A 250 ml. chemostat was used to measure the yield (Y) and saturation (K_s) constants respecting vitamin B_{12} in the chrysomonad *Monochrysis lutheri*. The notations below are after Herbert, D., Elsworth, R. & Telling, R. C. ((1956), *J. gen. Microbiol.* **14**, 601).

Forty-five steady-state measurements of cell numbers ($\bar{x}$) and flow (D), with vitamin B_{12} limiting ($s_R = 10$ $\mu\mu$g./ml. in the input) gave a linear regression of $D = 0{\cdot}8 - 0{\cdot}23\bar{x}$ for flow rates between 0·2 and 0·5 vol./day. The flow rate for maximum output (D_M) was therefore 0·40 vol./day. μ_R (i.e. the specific growth rate μ when $s = s_R$) in batch culture was 0·645/day. Hence K_s, computed from $D_M/\mu_R = (K_s/s_R + 1)(1 - \sqrt{[K_s/(K_s + s_R)]})$, was 6·0 $\mu\mu$g./ml. As a check, assay of the outflow concentration ($\bar{s}$) gave $\bar{s} = 2{\cdot}7$ $\mu\mu$g./ml. when $D = 0{\cdot}39$ and $\bar{x} = 1{\cdot}86 \times 10^6$ cells/ml.; and $\bar{s} = 6{\cdot}5$ when $D = 0{\cdot}55$ and $\bar{x} = 1{\cdot}23 \times 10^6$ cells/ml. The values of K_s, computed from $K_s = (\mu_R - D)/(D/\bar{s} - \mu_R/s_R)$, were 3·21 and 4·7 $\mu\mu$g./ml., respectively, both values lying within the 95% fiducial limits of the previous estimate. Similarly, the values of Y, computed from $Y = \bar{x}/(s_R - \bar{s})$, were 0·26 and $0{\cdot}25 \times 10^6$ cells/$\mu\mu$g., respectively.

Possible reasons will be discussed for the disagreement of these results with previous batch culture estimates (Droop, M. R. (1961), *J. mar. biol. Ass. U.K.* **41**, 69), when $K_s < 0{\cdot}1$ $\mu\mu$g./ml., and $Y = 0{\cdot}8 \times 10^6$ cells/$\mu\mu$g). Chemostat measurements do not take into account sequestration of the vitamin, for which there is some evidence, and the saturation constant could be much lower than that measured. Interpretation of the role of vitamin B_{12} in marine ecology may thus now have to take into account variation of apparent K_s with population size and of Y with specific growth rate.

Bacterial Chemostat-Enrichments from Sea water. By H. W. JANNASCH (*Woods Hole Oceanographic Institution, Woods Hole, Mass. U.S.A.*)

The selective factors of the homogenous continuous culture system have been used for enriching various heterotrophic bacteria from natural waters (Jannasch, H. W. (1965), *Zbl. Bakt.* Suppl. vol. 1). In recent experiments aboard a research vessel, unsterile sea water from a teflon-lined pipe was passed through a number of chemostats (1 l. capacity). Simultaneously, a sterile medium containing a limiting substrate (glycerol, lactate, or acetate) as well as phosphate and ammonia in excess was metered in at a much slower rate than the sea water. A variety of strains were enriched by selecting substrate concentrations within the range of 0·1–100 mg./l. in the reservoir and dilution rates within the range of 0·05–0·5 hr^{-1}. Samples from the enrichment cultures were streaked on agar plates at intervals of half of the retention time. The change in the proportion of colony types was recorded, and isolates were collected. The enrichments could be reproduced in the laboratory under identical cultural conditions except that sterile filtered sea water inoculated with an unsterile sample was used. Experiments with a multi-stage chemostat showed that, with the substrates used, no satisfactory enrichments could be achieved beyond the second stage. The purpose of enriching and isolating bacterial strains of high affinity for a substrate (i.e. low substrate saturation constant) was to study the metabolic activities of these forms as representatives of the marine autochthonic micro-flora.

Classification of Bacillus spp. from Marine Sediments. By G. J. BONDE (*Institute of Hygiene, University of Aarhus, Denmark*)

During an investigation of pollution bottom and water samples were collected from 184 stations. The main programme comprised an examination of Gram-negative rods and anaerobic spore-formers, but 95 Bacillus strains comprising 16% of the aerobic isolates, were also collected. Seventy-two of these Bacillus strains were subjected to 64 tests giving for classification 145 characters. Identification was attempted according to the keys of Gibson, T. ((1938), *Soc. Agric. Bact. Proc. Abstr.* 43–4); of Smith, N. R., Gordon, R. E. & Clark, F. E. ((1952), *Aerobic Sporeforming Bacteria, Agriculture Monograph*, no. 16, U.S. Dep. of Agric.) and of others. Only the Gibson-key permitted a grouping of all strains.

Data from 31 of these sedimental-strains together with that from 14 strains of *Bacillus* from spoilt foods were examined by computers using cluster analysis and by grouping according to overall similarity.

Three groups were distinguished joining to a common stem at the 67% S-level. When compared with the key classification the largest group of 11 were cereus-like, a group of 8 were sphaericus-like, and a small group of 3 consisted of strains of the licheniformis group. The remaining strains were either intermediates between the three groups, or joined the common stem at a lower level. The marine strains were all placed in the two main groups, or were intermediate between them.

Some Aspects of the Catabolism of the Marine Bacterium, *Pseudomonas natriegens*. By R. G. EAGON and H. W. CHO (*Department of Bacteriology, University of Georgia, Athens, Georgia, U.S.A.*)

Pseudomonas natriegens has been shown to produce sufficient acid in carbohydrate broth when growing under aerobic conditions to give a positive methyl red test and a pH of 4·5. Production of gas could not be detected when *P. natriegens* was cultivated in fermentation tubes. The acids produced were identified as acetic, pyruvic and lactic acids. Resting cells, however, converted glucose transiently to the α-keto acids, pyruvic and α-ketoglutaric, with yields of over 40%. When glucose was oxidized by resting cells, only 42% of the theoretical O_2 uptake was observed. Radio-respirometric experiments revealed that 92% of the glucose actually catabolized by growing cells was dissimilated via an aerobic Embden–Meyerholf pathway, whereas only 8% was dissimilated via the hexose monophosphate pathway which appeared to be limited by the supply of $NADP^+$. Both D- and

L-lactic dehydrogenases were detected and were shown to be flavin-linked enzymes. The pyruvic dehydrogenase reaction sequence appeared to be able to utilize $NADP^+$ as co-factor. Isocitric and glutamic dehydrogenases were also shown to be $NADP^+$ linked while malic and α-ketoglutaric dehydrogenases were NAD^+-linked. Succinic dehydrogenase appeared to be a flavoprotein. The specific activities of these enzymes were higher in extracts from nutrient broth-grown cells than from glucose-grown cells, indicating depression of the tricarboxylic acid cycle by the glucose. Isocitric lyase, but not malate synthetase, was detected in extracts of glucose-grown cells. Malate synthetase was induced by cultivation on acetate when isocitric lyase was increased by more than sixfold. These results are consistent with the hypothesis that the rate of $NADP^+$ supply plus depression of the tricarboxylic acid cycle by glucose results in the accumulation of acidic end products.

SYMPOSIUM ON MARINE MICROBIOLOGY

Marine Bacteria especially *Micrococcaceae*. By J. Brisou, C. Tysset, Y de Rautlin de la Roy and R. Curcier (*École Nationale de Médicine et de Pharmacie de Poitiers, University de Poitiers, France*)

Marine bacteria have wide and varied physiological properties despite the low concentration of nutrient organic matter in the sea. The physiological activity of marine bacteria can be examined either by studying the biochemical properties of the isolated bacteria, or by studying their enzymic activities under natural conditions. Their biochemical properties were deduced from more than 120,000 experimental observations. 75–80% of the isolated samples produced proteolysis, reduction of nitrates to nitrites and had an action on sugars and fats. Indole and hydrogen sulphide production (more frequently encountered in Mediterranean than in Atlantic waters) were ecologically related as were ureolysis and chitinolysis.

With regard to nutrient organic matter, 400 analyses of Atlantic, English Channel and Mediterranean waters showed the content of organic matter to be on the average 4·15 mg./l. rising to 10 mg./l. near beaches and ports. These concentrations are probably the minimum which will allow the growth of heterotrophs in natural sea water. Native bacteria such as *Vibrio* and *Pseudomonas* spp. will grow in sea water containing 8–10 mg./l. organic matter. On the other hand, heterotrophic mesophiles and pathogenic bacteria such as *Staphylococcus aureus* or *Escherichia coli* cannot grow under these poor nutritional conditions. Consequently they are seldom encountered in sea water except on fish, shell fish, plankton and in some sediments. However, although sea water is too poor in nutrients by a factor of 100–500 to allow pathogenic bacteria to grow, the latter can survive for a long time, retaining their biochemical properties and in fact they are good 'tracers' of currents and of pollution. In the non-growing, 'quiescent' form they are quite resistant to bactericidal agents. When adsorbed on organic or inorganic particles or on plankton they can grow and by selection, mutations, etc., can give rise to new varieties.

Marine bacteria generally are Gram negative; only 0·5–5% are Gram positive and hence it might be of interest to report some recent work on about 200 *Micrococcaceae* comprising 2% of our total isolates from sea water.

The bacteria were isolated either on the usual media or on selective substrates such as tellurite and azide, and, after purification, were submitted to a series of tests including respiratory type; action on bile salts, on skim milk containing 0·1% methylene blue, on sugars and on casein; growth at 45°; proteolysis and haemolysis; and coagulase activity and phage sensitivity for positive strains. As a result of these tests, the micrococci, isolated from three main sources plankton, open sea and shore waters, have been arranged into a number of specific or physiological groups. Most species are non-proteolytic and do not reduce nitrates. Some ferment carbohydrates, others are non-proteolytic but reduce nitrates.

These results are different from those obtained with Gram-negative bacteria in which proteolytic and nitrate-reducing types predominate. The sea-water micrococci have been well documented; ZoBell & Upham ((1944), *Bull. Scripps. Inst. Oceanogr.* **5**, 239), Kriss ((1963), *Marine Microbiology* (*Deep Sea*), Oliver and Boyd, Edinburgh and London),

Wood ((1952), *J. gen. Microbiol.* **6**, 205), Cviic (*Ribarstveno-bioloska ekspedicija m/b 'Hvar'. Izvjecsa*, Rep. 4, no. 1), Anderson ((1962), *J. appl. Bact.* **25**, 362) and Brisou ((1955), *Microbiologie du milieu marin*, Flammarion, Paris). Anderson considers it too early yet to suggest a classification for these bacteria; but we think we have obtained a fairly composite picture which agrees with the actual taxonomic position.

On the whole, all micrococci grow well in sea water except for a few strains of *M. afermentans*, *M. luteus*, *M. candidus* and *M. aquivivus*. The main species found have been *M. luteus* (30 strains), *M. candidus* (26), and *Strep. faecalis* (16). *M. luteus* predominates in the open sea off Dakar, while off La Rochelle, *M. candidus* and *Strep. faecalis* occur most frequently. Authentic strains of *Staph. aureus* are very rarely found in sea water even near the shore, while *Strep. faecalis* is quite numerous on the beaches and is good evidence of pollution.

From this study we propose a determinative scheme for the marine micrococci based mainly on their physiological characteristics and respiratory types. So far, about 200 species have been described in the literature, but since the original descriptions of many of these are very incomplete, they have been omitted from our scheme, thus simplifying the systematics. We propose one key which comprises 44 species and all the isolated bacteria are classified by a simple set of tests.

Marine Sedentary Bacteria. By G. D. Floodgate (*Marine Science Laboratories, University College of North Wales, Menai Bridge, Anglesey*)

The ability of aquatic bacteria to adhere to the solid particles usually found suspended in natural waters is well established (Henrici, A. T. (1933), *J. Bact.* **25**, 277; ZoBell, C. E. (1946), *Marine Microbiology*, Chronica Botanica; Kriss, A. E. (1962), *Marine Microbiology (Deep Sea)*, Oliver and Boyd). The use of the term 'periphytic' by Henrici to describe this phenomenon is unfortunate on several counts and the terms 'sedentary' or 'settled' are preferred here. The ability to settle and grow on solid surface is probably of great importance in the mineralization cycle in the sea, particularly in coastal waters.

The easiest way to demonstrate the phenomenon is to allow microscope slides to remain suspended in water for periods varying from about 1 hr to several days. Bacteria will be found adhering to the glass as single rod-shaped cells, as microcolonies or as long filaments, including some containing spore-like structures. Cocci appear only rarely. The degree of colonization depends upon the length of submersion and the bacteriological condition of the water mass. The method has been used extensively by Kriss in the major oceans, and he has demonstrated bacterial settlement down to a depth of several thousand metres. In many seas he observed large numbers of an unusual organism which consisted of a long, slender thread, one end of which was attached to the slide, while the other end formed a bulbous head. Kriss created the class Krassilnikoviae for these organisms. The validity of these observations has been challenged, however, and it has been suggested that the alleged organisms were, in fact, detached colloblasts of ctenophores. Attempts to observe organisms that clearly belong to the Krassilnikoviae on slides submerged in the Menai Straits have failed; but careful observation suggests two organisms growing in close proximity may produce an appearance not unlike that of the Krassilnikoviae as illustrated by Kriss.

Another form of bacterial settlement recently described by Christie & Floodgate may be confined to turbid, fast-flowing water, such as the Menai Straits. In this case small, fragile, branching structures, not unlike certain hydroids in appearance, have been found growing on plastic panels and glass slides. They are found only from October through to March. In spring and summer, the structures are overgrown by fouling macro-organisms. The structures are composed of mineral crystals, organic detritus and bacteria. The latter may be partly responsible for maintaining the integrity of the structure by producing sticky capsular materials, though the mutual adhesion of clay particles may also contribute. The presence of a greater concentration of calcite in the structures than in the surrounding water suggests that the bacteria may precipitate calcium carbonate *in situ* from the sea water by a local pH change, caused by ammonia produced from proteinaceous material.

Field observations such as these have suggested laboratory experiments. Past workers (e.g. ZoBell, C. E. & Anderson, D. Q. (1936), *Biol. Bull. mar. biol. Lab. Woods Hole*, **71**, 324; Harvey, H. W. (1941), *J. mar. Biol. Ass. U.K.* **25**, 225) have shown that when natural sea water, in which dissolved nutrients are present only in very low concentration, is allowed to stand, there is a rapid rise in bacterial numbers, and that one important factor governing the rate and amount of growth is the surface area of the vessel. These results were attributed to the absorption of nutrients on the glass to which the bacteria also attached themselves. A similar relationship between dilute nutrient supply and growth on the walls of the vessel has been shown for *E. coli* by Heukelekian & Heller ((1940), *J. Bact.* **40**, 547). Bacterial settlement has been further investigated in this laboratory using a pure culture of a Gram-negative rod isolated from the Menai Straits. Low concentrations of washed cells of the organism have been suspended in artificial sea water containing no organic material. Acid washed glass surfaces and surfaces on which nutrient material had been pre-adsorbed were submerged for varying periods in the suspension. Some bacteria settled on clean slides in the absence of organic material. Settlement on the surfaces with adsorbed nutrients showed no evidence of chemotaxis; cells were observed both to settle on the glass and to leave it again. However, once the organism had finally settled the adsorbed nutrients apparently affected the rate of cell division.

Some Recent Studies on Marine Bacteriophages. By Perviz N. Chaina (*Torry Research Station, Aberdeen*)

Large numbers of bacteriophages have been isolated and studied, and they are abundant in soil, sewage, decaying vegetable matter and inland waters. However, knowledge of their occurrence and activity in the sea is very fragmentary. The present paper records a study of the bacteriophages present in Indian waters, and in the North Sea.

In the Indian work, sea-water and mud samples were collected at various depths from the surface down to 800 m. The area investigated was between latitudes 16° 18′ N. to 20° 42′ N. and longitudes 62° 52′ E. to 72° 55′ E. The temperature of the sea water varied from 10·8° to 31° and that of mud samples from 27·3° to 29·3°. 'Lemco' agar, sea-water 'Lemco' agar and ZoBell's medium 2216 were used for the isolation of bacteria and sea-water 'Lemco' agar to isolate and study the bacteriophages. After preliminary isolation of the bacteria, attempts were made to isolate bacteriophages by direct and indirect methods. Using incubation at room temperature (25°–28°), a total of 629 cultures of bacteria were isolated, of which 10 were sensitive to bacteriophages. The sensitive cultures belonged to the following genera: *Pseudomonas* (5), *Flavobacterium* (2), *Achromobacter* (2) and *Vibrio* (1). Identification was carried out according to Bergey's *Manual of Determinative Bacteriology* (7th ed.) and ZoBell & Upham ((1944), *Bull. Scripps Inst. Oceanogr.* **5**, 239–92).

The bacteria and the bacteriophages were mildly halophillic and could tolerate a salt concentration up to 17%. The bacteriophages were more stable to heat than their hosts and could tolerate 50° for 30 min. All bacteriophages were killed at 60° for 15 min. but were viable at 60° for 2–5 min. All were active at pH 7·3–7·9, one showing slight activity at pH 7·0, two at pH 8·0 and one at pH 8·5. Compared with bacteriophages isolated by Spencer from the North Sea it would appear that those from tropical waters are the more resistant to heat.

The marine bacteriophages were tested against 25 species of bacterial fish pathogens; only one was active against one bacterial species, namely, *Vibrio ichthyodermis*.

Some recent electron microscope studies of bacteriophages isolated from the North Sea will also be discussed.

Experimental Studies on Some Representatives of the Haptophyceae. By A. D. Boney (*Department of Botany, University College of Wales, Aberystwyth*)

The class Haptophyceae has been created in recent years to include those organisms, originally classified in the Chrysophyceae, which, in their motile phase, bear paired acronematic flagella; in many instances these motile organisms also bear a specialized organelle,

the haptonema (Parke, M. (1961), *Brit. phycol. Bull.* **2**, 47; Christensen, T. (1962), Alger, *Botanik*, Bd. 2, no. 2, 178). Where these organisms bear scales they are either organic or of a calcarous nature (coccoliths). With a number of marine representatives of these motile coccolithophorids, growth in clonal culture without frequent subculturing produces benthic forms which, in their thallus condition, are similar to plants earlier described as separate entities in the Chrysophyeeae (e.g. *Apistonema*, *Chrysotila*, *Chrysonema*); (Stosch, H. A. v. (1955), *Naturwissenschaften*, **42**, 423; Stosch, H. A. v. (1958), *Naturwissenschaften*, **45**, 140; Pringsheim, E. G. (1955), *Arch. Mikrobiol.* **21**, 401; Parke, M. (1961), *Brit. phycol. Bull.* **2**, 47; Valkanov, A. (1962), *Rev. algol.* **6**, 220). A similar range of form of benthic phase has been observed in cultures of coccolithophorids isolated from various localities, including some from the coast of Wales. The benthic forms obtained from these coccolithophorids in culture have been compared with similar plants found in nature.

In fresh culture media these benthic phases release again the motile coccolithophorids. Studies have been made on the release of motile cells from the benthic phase under experimental conditions simulating the environmental factors in habitats characteristic of the benthic phase in nature. These have shown that release of motile cells can take place over a wide salinity range (0·3–9%), and that benthic cells can survive protracted immersion in media of high salinity (9–24%) without releasing motile cells, and still release them on re-immersion in normal media. A possible selection of 'physiological races' seems to occur when coccolithophorids are cultured over protracted periods in media of different salinity. Together with the euryhaline nature of coccolithophorid release, the benthic phase appears well adapted to changes in temperature and exposure to air of different humidities. Release of coccolithophorids has been obtained in a pH range of 3·4–10; at pH values lower than 5·7, motile cells were obtained lacking coccolith cover. Lowered salinities similarly result in a release of cells lacking coccoliths. Studies have been made on the growth in culture of these motile cells which lack coccoliths.

The coccolithophorids are well known constituents of the marine microplankton. The benthic forms (as distinct entities) have been described from estuarine and intertidal habitats. The results of these experimental studies have indicated that benthic forms of marine coccolithophorids, obtained in clonal culture, are well adapted to habitat conditions of this nature. These observations give added weight to the recent inclusion of these benthic algae (*Apistonema*, *Chrysonema*, *Chrysotila*) in the Haptophyceae (Parke, M. (1964), *J. mar. biol. Ass. U.K.* **44**, 518).

The Effect of Moderate Temperature on a *Vibrio marinus*, an Obligate Psychrophilic Marine Bacterium. By R. Y. Morita (*Departments of Microbiology and Oceanography, Oregon State University, Corvallis*)

Numerous obligate psychrophilic bacteria, until recently thought to be non-existent, have been isolated from the sea below the thermocline and the Arctic area.

Vibrio marinus ATCC 15381, which has a maximum growth temperature of 20° and an optimum growth temperature of 15°, was selected for studies to determine why exposure to temperatures between 20° and 30° killed the organism. Inactivation of the metabolic mechanism involved in oxygen uptake either endogenously or in the presence of glucose occurred when the cells were heat shocked at temperatures between 20° and 30°. The data suggested that biochemical lesion(s) had occurred in a metabolic pathway involved in oxygen uptake.

When the cells were subjected to moderate temperatures leakage of cellular materials occurred. After the heat treatment of the cells, the samples were centrifuged at 0°, decanted, and the supernatants stored on ice. The supernatants were assayed for protein, RNA, DNA, and amino acids by the biuret, orcinol, diphenylamine and ninhydrin methods respectively. After protein determinations had been made, the supernatants were deproteinized by heating adding 10% (w/v) TCA, and clarifying by filtration. The subsequent supernatant contained malic dehydrogenase, glucose-6-phosphate dehydrogenase as well as other protein material, RNA, DNA, and amino acids.

Malic dehydrogenase in the organism was stable at temperatures from 1° to 14°. Increasing the temperature beyond 14° for 60 min. inactivated the enzyme in the cells. In cell-free extracts in 0·2M tris-sulphate nearly 95% of the malic dehydrogenase was inactivated in 10 min. at 30°. Exposure to moderate temperatures inactivated partially purified malic dehydrogenase. The addition of ammonium sulphate protected the malic dehydrogenase against the effects of moderate temperature. Other salts did not stabilize the enzyme. When cells were subjected to moderate temperature in the presence of ammonium sulphate (7·5%) leakage of malic dehydrogenase was decreased, presumably by the stabilization of the cellular membrane.

The above data suggest that moderate temperatures destroy the control of permeability of the cell.

The Role of Inorganic Ions in the Physiology of Marine Bacteria. By R. A. MacLeod (*Department of Microbiology, Macdonald College of McGill University and Marine Sciences Center, McGill University, Montreal*)

The highest bacterial counts on sea water or marine materials are obtained if the plating medium is prepared with sea water or with 3% NaCl (see ZoBell, C. E. (1946), *Marine Microbiology*, Chronica Botanica Co., Waltham, Mass.). Studies using chemically defined media have shown that the need for sea water reflects a requirement of marine bacteria for particular inorganic ions. Most notable is a highly specific requirement for Na^+ which has been detected in all marine bacteria (see MacLeod, R. A. (1965), *Bact. Rev.* **29**, 9). Na^+ dependent transport mechanisms have been found in marine bacterial cells (Drapeau, G. R. & MacLeod, R. A. (1963), *Biochem. Biophys. Res. Commun.* **12**, 111). The quantitative requirements of these mechanisms for Na^+ were examined using non-metabolizable analogues of metabolizable substrates and were found to vary with the substance being transported. These variations in requirements for transport correlated well with the amounts of Na^+ needed for growth when the corresponding metabolites served as sole sources of carbon in the medium (Drapeau, G. R. & MacLeod, R. A., unpublished). It was thus concluded that in the case of at least one marine pseudomonad the Na^+ requirement for growth represented a requirement for Na^+ for the transport of substrates into the cell.

Many marine bacteria lyse when suspended in distilled water. Studies with isolated envelopes of a marine pseudomonad showed that at low salt concentration both non-diolysable and dialysible material appeared in solution. This occurred with both heated and unheated preparations suggesting that the breakdown of the envelope was not enzymic. At higher temperatures, higher salt concentrations were required to prevent the loss of soluble material from the cell envelopes. The non-diolysible material appearing in solution at low salt concentration was found to contain among other things, glucosamine, muramic acid and diaminopimelic acid. Electron micrographs of thin sections of the envelopes revealed that at low salt concentration the outer layer of the cell wall remained intact. These findings are consistent with the theory that in this marine pseudomonad the rigid mucopeptide layer is composed of units held together by cross-linkages between polyanions on adjacent units. The units would be able to come close enough together to form a continuous layer only if the negative charges on the polyanions were screened by the cations of a salt (Buckmire, F. L. A. & MacLeod, R. A., *Can. J. Microbiol.*, in the Press).

A third function for inorganic ions in the life of marine bacteria has recently come to light. This is a role in maintaining intracellular solute concentrations and was first observed with a marine pseudomonad (Drapeau, G. R. & MacLeod, R. A. (1965), *Nature*, **206**, 531). The results have been extended to include studies with a Photobacterium (Srivastava, V. & MacLeod, R. A., unpublished). Cells of both species were allowed to accumulate [^{14}C]-α-amino isobutyric acid, a non-metabolizable substrate that can be concentrated by the cells. When the cells were removed from the incubation medium and resuspended in various salt solutions, the labelled compound was retained by the cells in some suspensions while in others it was lost to the medium. Viability studies conducted with the *Photobacterium* species showed that salt solutions permitting retention of the labelled compound also maintained the viability of the cells. Conditions which did not permit the retention of the

labelled compound, however, did not necessarily lead to loss of viability of the cells. These findings suggest that a critical solute whose loss leads to rapid death of the cells is retained under conditions which are somewhat different from those required to maintain the intracellular level of α-amino-isobutyric acid.

Gaffkaemia, the Blood Disease of Lobsters. By P. C. Wood (*M.A.F.F., Fisheries Laboratory, Burnham-on-Crouch, Essex*)

The death of lobsters associated with infection by a tetrad-forming micrococcus was first reported by S. F. Snieszko & C. C. Taylor ((1947), *Science*, **105**, 509) and since that time the disease has been identified in Eire, Holland and Canada. The condition is typified by the sudden death of large numbers of lobsters during commercial storage in tanks of sea water. In 1962 the disease was observed in storage tanks in southern England and deaths occurred for two periods of several weeks. The lobsters became weak, and died without external symptoms, except that the blood of damaged lobsters failed to clot. Examination of the blood revealed large numbers of tetrad-forming encapsulated micrococci which have previously been described as *Gaffkya homari* (Aaronson, S. (1956), *J. gen. Microbiol.* **15**, 478), *Pediococcus homari* (Deibel, R. H. & Niven, C. F. (1960), *J. Bact*, **79**, 175) and *Aerococcus* sp. (Coster, E. & White, H. R. (1964), *J. gen. Microbiol.* **37**, 15). The organism was also isolated from the water in the storage tanks, and from the walls and equipment. The strain isolated was compared with those recovered from dead lobsters in America, Eire and Canada. All strains had marked cultural and biochemical similarities, producing white colonies of 1 mm diameter on blood agar base (Oxoid Ltd) and a zone of β-haemolysis on sheep blood agar. All were Gram-positive cocci which could ferment glucose, sucrose, lactose and maltose in 24 hr, and mannitol in 48 hr; they were unable to produce indol, acetyl methyl carbinol, catalase and gelatinase. Although there may be some dispute about the precise nomenclature of these strains, there can be no doubt about the close similarity of strains isolated from lobsters from a variety of sources.

There is evidence that the organism is present in healthy lobsters when they are taken from the sea. Over 50% of lobsters examined by J. E. Stewart and J. F. MacDonald in Canada ((1962), *Circular No.* 9, *Fish Res. Bd. Can.*) were infected with *Gaffkya*-like organisms and the organism has also been isolated from two lobsters taken from the North Sea. A survey of British lobster stocks was indeterminate, for although strains were isolated which were culturally and biochemically identical with the known pathogenic organisms, injection of broth cultures into healthy lobsters failed to cause infection. This may have been due to the loss of virulence of cultured organisms.

Taxonomic Studies of Strains of Marine Luminous Bacteria. By Margaret S. Hendrie, W. Hodgkiss and J. M. Shewan (*Torry Research Station, Aberdeen*)

Luminous bacteria are not uncommon in the marine environment and even as early as 1920, many species had been described and assigned to no fewer than 15 different genera.

In 1889, Beijerinck (*Archs néerl. Sci.* **28**, 401) proposed that all luminous bacteria be placed in the genus *Photobacterium*, but in 1916 (*Folia microbiol.* **4**, 15) he recognized that there was already much confusion in their nomenclature. At present almost 100 generic and specific epithets have appeared in the literature and the resulting confusion is dealt with in the 7th edition of *Bergey's Manual* (1957).

Breed & Lessel ((1954), *Antonie van Leeuwenhoek*, **20**, 58) reviewed the classification of luminous organisms and proposed that they be placed in the genera *Photobacterium* (a genus within the family Pseudomonadaceae) and *Vibrio*. Spencer ((1955), *J. gen. Microbiol.* **13**, 111) also pointed out the affinities of some of these organisms to *Vibrio* and to *Aeromonas*.

The present position in *Bergey's Manual* is that four species (*Photobacterium phosphoreum*, *P. pierantonii*, *P. fischeri* and *P. harveyi*) are placed in the genus *Photobacterium* and four species are placed in the genus *Vibrio* (*V. albensis*, *V. indicus*, *V. luminosus* and *V. pierantonii*). The type species, *P. phosphoreum*, is described as a coccobacillus which is

either not motile, or only shows an occasional motile cell. All the species having a motile phase are described as possessing one or more polar flagella.

Our present studies have been carried out on 50 strains of luminous bacteria in the National Collection of Marine Bacteria. All the strains are Gram-negative rods which, although sharing the common feature of luminescence, exhibit morphological differences which, to a lesser extent, are reflected in their biochemical activities.

Electron microscopy has shown three main types of cell morphology: (*a*) non-motile coccobacilli without flagella; (*b*) motile rods with polar flagella, and (*c*) peritrichously flagellate rods. The last group show a unique type of flagellation. In addition to fine lateral flagella each cell possesses a much thicker flagellum at one or both poles. Although the two types of flagella were described by Johnson *et al.* ((1943), *J. Bact*, **46**, 167) in Johnson's original strain of *Photobacterium harveyi*, this characteristic appears to have been overlooked in subsequent literature. The thicker, polar flagellum of *P. harveyi* shows a distinct sheath, a structure analogous to that described in some *Vibrio* spp. by Follett & Gordon ((1963), *J. gen. Microbiol.* **32**, 235) and Ogasawara & Kuno ((1964), *Nagoya J. med. Sci.* **26**, 99 and 109). Some strains of *P. harveyi* also possess fimbriae, organelles which we have not previously detected in marine micro-organisms.

Starch-gel electrophoresis of the esterases and catalases of all the strains examined show three main types of pattern, one of which shows some affinity to the genus *Vibrio*.

Physiological studies indicate that the optimum conditions for luminescence are variable, but the majority of strains do not grow in the absence of NaCl and most prefer 2–3% NaCl. Usually the temperature range for growth is limited and, although some strains grow at 37°, most prefer temperatures between 5° and 30°. All strains ferment glucose. Some are oxidase positive and sensitive to 2,4-diamino-6,7-di-isopropyl-pteridine which indicates that these strains may be correctly assigned to the genus *Vibrio* (Shewan *et al.* (1954), *Nature, Lond.* **173**, 208; (1960), *J. appl. Bact.* **23**, 379).

The taxonomic position of the remaining types is more difficult to assess but possible assignments will be discussed.

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